195

ACTA PHYSIOLOGICA LATINOAMERICANA

Vol II - N.º 4

Diciembre 1952

ASOCIACION CIENCIA E INVESTIGACION

Buenos Aires - Argentina

SINTESIS DEL P-NITROFENILSULFATO POR EL HIGADO DE RATA *

R. H. De Meio

(Department of Biochemistry. Jefferson Medical College. Philadelphia, Pennsylvania, U. S. A.)

En publicaciones anteriores ([4, 5]) hemos estudiado diversos aspectos de la conjugación del fenol (síntesis de fenilsulfato) por preparados de hígado de rata. Es sabido que además del fenol ordinario, muchos otros fenoles y entre ellos el p-nitrofenol dan lugar a la formación de ésteres sulfúricos cuando se administran a diversos animales ([15]).

Para comprobar si el resultado obtenido "in vivo" podía confirmarse "in vitro" y si el sistema enzimático que sintetiza al fenilsulfato era el mismo que para el p-nitrofenilsulfato, es que emprendimos este estudio. Además, de ser posible la utilización del p-nitrofenol como sustrato para el estudio de la síntesis de ésteres sulfúricos, la técnica a utilizar se simplificaría considerablemente.

Métodos

El hígado era extraído de ratas blancas macho inmediatamente después de sacrificadas y sumergido en un medio de la siguiente composición: 78 ml ClK 0.154 M; 2 ml SO_4Mg 0.154 M; 20 ml de mezcla reguladora de fosfato de sodio 0.1 M y de pH 7 (a pH 6.2 no se observaba actividad y disminuía a pH 7.6); succinato de sodio 0.01 M; adenosina-5-fosfato 0.001 M y las concentraciones de p-nitrofenol que se indican más adelante. Mediante el empleo del homogeneizador de Potter-Elvehjem se preparaba un homogeneizado que contenía 5 ó 10 por ciento de hígado (5 ó 10 g en 100 ml. de medio de suspensión). El procedimiento utilizado para el fraccionamiento de este homogeneizado se describe más adelante. El prepa-

* Este trabajo se realizó con la ayuda de una subvención (contrato NONR 229 [00]) del "Office of Naval Research" del gobierno de los Estados Unidos de N. A. y fué presentado en parte al 2º Congreso Internacional de Bioquímica ([6]) reunido en París (21-27 de julio de 1952).
Recibido para su publicación en Noviembre 16, 1952.

rado se mantenía entre 0 y 5° C durante todo el proceso que precedía a la incubación. Cuatro mililitros de la preparación, colocados en Erlenmeyers de 50 ml, se incubaban a 37.5° C en atmósfera de oxígeno.

Para la determinación del p-nitrofenol adaptamos la técnica descripta por Huggins y Smith (12), basada en el conocido hecho de que sólo el p-nitrofenol libre da coloración amarilla en medio alcalino. La desproteinización del homogeneizado se efectuaba diluyendo los 4 ml. de preparado a 13 ml. con agua destilada y agregando 1 ml de SO_4Zn al 10 por ciento, seguido de 1 ml de NaOH 0.5 N de acuerdo a la técnica de Somogyi (14). Hallamos que con la técnica de Somogyi, se obtenían testigos (sin p-nitrofenol) que manifestaban menor absorción luminosa que los obtenidos con cualquier otra técnica de desproteinización. Se separaba el precipitado por centrifugación y en el sobrenadante se determinaba el p-nitrofenol como se indica a continuación. Para la determinación del p-nitrofenol libre, una parte alícuota del sobrenadante se llevaba a 7.5 ml con agua destilada. Agregando luego 2.5 ml de NaOH 2N, se efectuaba la lectura con el filtro 420 en el fotocolorímetro de Evelyn, usando como testigo un desproteinizado desprovisto de p-nitrofenol y tratado en la misma forma. El p-nitrofenol total se determinaba en otra parte alícuota, que se sometía a hidrólisis colocándola en baño maría a ebullición durante 15 minutos, en presencia de ClH 0.3 N. Luego de enfriada, se neutralizaba la acidez con NaOH y se procedía en la misma forma indicada para la determinación del p-nitrofenol libre. La concentración de p-nitrofenol se obtenía de un gráfico preparado con soluciones de concentración conocida. La diferencia entre el p-nitrofenol total y el libre da el combinado, el que por las condiciones de hidrólisis puede identificarse con el p-nitrofenilsulfato (8). Los resultados se expresan en microgramos de p-nitrofenol en los 4 ml de preparado. Con el método empleado se recuperaba un 75 por ciento del p-nitrofenol agregado a los controles.

Influencia de la concentración de p-nitrofenol y de sulfato. — El tipo de preparado y el medio utilizado, así como las condiciones de incubación se describen más arriba. En la tabla 1 se presentan los resultados obtenidos al estudiar el efecto de la concentración de p-nitrofenol y de sulfato sobre el proceso de conjugación. Puede verse que, a la concentración de 0.9×10^{-4} M, un 66 a 78 por ciento del p-nitrofenol es transformado en p-nitrofenilsulfato por homogeneizado de hígado al 5 ó 10 por ciento. La ausencia de sulfato en el medio de suspensión reduce la conjugación a un 5 por ciento. En este sentido el p-nitrofenol se comporta como el fenol (5). A concentraciones más altas de p-nitrofenol, el porcentaje combinado se reduce, siendo del 30 a 35 por ciento para una concentración de 1.8×10^{-4} M y nulo para 3.6×10^{-4} M. Aquí se observa una diferencia fundamental con el fenol, el que no produce inhibición alguna aún a concentraciones del orden de 5×10^{-4} M. Una posible explicación del comportamiento del p-nitrofenol, reside en la propiedad que posee este compuesto de desacoplar el proceso de fosforilación (1, 13). Que esta hipótesis es aceptable, lo demuestra la acción inhibidora de la gramicidina (agente que obra de manera análoga) sobre la formación de p-nitrofenilsulfato, que mencionamos más adelante.

Influencia del fenol. — Como era de esperar, la adición de fenol inhibió parcialmente la formación de p-nitrofenilsulfato. A la concentración de 2.7×10^{-4} M la conjugación del p-nitrofenol se redujo en un 21 por ciento. En la tabla 2 se incluyen valores representativos correspondientes a cuatro experimentos. El efecto del fenol se debe, con toda probabilidad,

TABLA 1

Efecto de la concentración de p-nitrofenol y de sulfato sobre la formación del p-nitrofenilsulfato por homogeneizado de hígado de rata.

Medio: 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M, pH 7, succinato de sodio 0.01 M, AMP 0.001 M.

Homogeneizado 5 ó 10 por ciento; 4 ml en cada Erlenmeyer; incubado durante una hora a 37.5° C en atmósfera de O_2.

Exp.	Concentración de p-nitrofenol	p-Nitrofenol combinado		Homogeneizado	Observaciones
		μg	por ciento		
1	0.9×10^{-4} M	35.5	71	10 %	
	0.9×10^{-4} M	33.0	66	10 %	
	0.9×10^{-4} M	39.2	78.4	5 %	
	0.9×10^{-4} M	29.1	58.2	5 %	
	0.9×10^{-4} M	2.7	5.4	5 %	Sin sulfato (*)
2	1.8×10^{-4} M	33.5	33.5	10 %	
	1.8×10^{-4} M	30.0	30	10 %	
	3.6×10^{-4} M	—	0	10 %	

(*) El SO_4Mg en el medio fué sustituído por la cantidad equivalente de $MgCl_2$.

a que compite con el p-nitrofenol por el sistema enzimático que sintetiza a estos ésteres sulfúricos. Un mayor número de experimentos serán necesarios para determinar con certidumbre la causa de este efecto y la relativa afinidad de los dos fenoles por el sistema enzimático.

Sustitución del sustrato oxidable. — Experimentos efectuados anteriormente con fenol ([5]), demostraron que el α-ketoglutarato podía ser sustituído por el succinato, sin que hubiera una modificación apreciable en la actividad de los preparados. Por esta razón es que tratamos de ver si la sustitución del succinato por otros sustratos oxidables (piruvato, fumarato, citrato) manifestaba el mismo comportamiento. Valores representativos de los resultados obtenidos se incluyen en la tabla 3, por la que puede verse que no se observan diferencias fundamentales.

TABLA 2

Efecto del fenol sobre la formación del p-nitrofenilsulfato por homogeneizado (al 5 por ciento) de hígado de rata.

Medio 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M; pH7; succinato de sodio 0.01 M; AMP 0.001 M; 4 ml de homogeneizado en cada Erlenmeyer; incubado por 1 hora a 37.5° C en atmósfera de oxígeno.

Concentración de p-nitrofenol	Concentración de fenol	p-Nitrofenol combinado	
		µg	Por ciento
0.9×10^{-4} M	—	32	64
0.9×10^{-4} M	2.7×10^{-4} M	21.6	43.2

Papel de la fosforilación. — Dado que el homogeneizado, para ser activo, requiere el agregado de adenosina-5-fosfato (AMP) y de un sustrato oxidable ([4]) y que la formación de fenilsulfato es inhibida por 2,4-dinitrofenol ([5]), es posible suponer que la formación del p-nitrofenilsulfato está también ligada a los procesos de fosforilación. En tal caso, agentes como la gramicidina que desacopla las fosforilaciones de los procesos oxi-

TABLA 3

Influencia de la sustitución del sustrato oxidable sobre la formación del p-nitrofenilsulfato por homogeneizado de hígado de rata.

Medio: 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M, pH 7, p-nitrofenol 0.9×10^{-4} M, sustrato oxidable 0.01 M, AMP 0.001 M. Homogeneizado 10 por ciento; 4 ml en cada Erlenmeyer; incubado por 1 hora a 37.5° C en atmósfera de 0_2.

Exp.	Sustrato oxidable	p-nitrofenol combinado	
		µg	Por ciento
1	Succinato	37.8	75.6
	Piruvato	31.3	62.6
2	Succinato	32.9	65.8
	Fumarato	36.7	73.4
3	Succinato	40	80
	Citrato	36.2	72.4

TABLA 4

Influencia de la gramicidina y del ATP sobre la síntesis del p-nitrofenilsulfato por homogeneizado (al 10 por ciento) de hígado de rata.

Medio: 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M, pH 7, p-nitrofenol 0.9×10^{-4} M. Cuatro mililitros en cada Erlenmeyer; incubado durante 30 minutos a 37.5° C.

Exp.	Fortificado con	Inhibidor	p-Nitrofenol combinado		Variación por ciento	Fase gaseosa
			μg	Por ciento		
1	Succinato 0.01 M + AMP 0.001 M	—	30.3	60.6		O_2
	Succinato 0.01 M + AMP 0.001 M	gramicidina 3×10^{-5} M	0.6	1.2	—98	O_2
2	Succinato 0.01 M + AMP 0.001 M	—	22	44		O_2
	Succinato 0.01 M + AMP 0.001 M	gramicidina 3×10^{-5} M	3.3	6.6	—85	O_2
3	Succinato 0.01 M + AMP 0.001 M	—	21.7	43.4		O_2
	Succinato 0.01 M + ATP 0.003 M	—	23	46		O_2
	ATP 0.003 M	—	0	0		N_2

dativos celulares ([3, 10, 11]) y obra en este sentido en forma análoga al 2,4 dinitrofenol, debe inhibir la síntesis del p-nitrofenilsulfato. Además, por las mismas razones ya expuestas, es aceptable admitir que el AMP es utilizado en la formación del adenosintrifosfato (ATP), el que serviría como fuente de energía para la síntesis. En este último caso, la adición de ATP al preparado, mantenido en anaerobiosis, debiera reemplazar a la combinación de AMP y sustrato oxidable.

Para los experimentos utilizamos metilol gramicidina*, derivado más soluble que la gramicidina misma. A pesar de su mayor solubilidad, fué necesario disolverla en alcohol etílico al 75 por ciento y agregar 0.03 ml de esta solución al preparado, para poder conseguir la concentración deseada (3×10^{-5} M)**. Este procedimiento ha sido utilizado por Cross y col. ([3]) satisfactoriamente. Además, nosotros hemos podido comprobar que la concentración de alcohol, obtenida en estas condiciones, no afecta para nada la síntesis del p-nitrofenilsulfato.

La solución de ATP fué preparada a partir de ácido adenosintrifosfórico *** de una pureza controlada de 70 por ciento, cuidadosamente neutralizado, y mantenido a 0° C hasta el momento de ser utilizado, para evitar su descomposición. Se ensayó el agregado de ATP a la preparación

TABLA 5

Efecto de la hipotonía y de la congelación sobre la síntesis del p-nitrofenilsulfato por homogeneizado (al 10 por ciento) de hígado de rata.

Medio: 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M, pH 7; succinato de sodio 0.01 M, AMP 0.001 M, p-nitrofenol 0.9×10^{-4} M. Cuatro mililitros en cada Edlenmeyer; incubado por 1 hora a 37.5° C en atmósfera de O_2.

Exp.	p-Nitrofenol combinado		Observaciones
	µg	Por ciento	
1	27	54	Medio isotónico
2	0	0	Medio hipotónico (*)
3	0	0	Congelación y descongelación repetidas

(*) Los 78 ml ClK 0.154 M del medio fueron sustituídos por el mismo volumen de agua destilada.

* Este producto fué gentilmente donado por los Laboratorios Wallerstein de Nueva York.

** La molaridad fué calculada considerando como peso molecular de la metilol gramicidina el de 2800 ([3]).

*** Obtenido de los Laboratorios Schwarz de Nueva York.

de una sola vez o en dos porciones (con 15 minutos de intervalo) para comprobar si así resultaba más eficaz, pero no se observó diferencia en el comportamiento. Es sabido que los tejidos poseen enzimas capaces de hidrolizar el ATP, y el agregado en porciones pudiera haber contribuído al mantenimiento de una concentración más alta de ATP, durante toda la incubación.

Los resultados consignados en la tabla 4 muestran que la gramicidina produce una inhibición del 80 al 98 por ciento, lo que va en apoyo de nuestra hipótesis. Por otra parte, los resultados negativos obtenidos con ATP en anaerobiosis, no se deben a una acción inhibidora de este compuesto, pues a la misma concentración (0.003 M), en presencia de succinato y atmósfera de oxígeno, produce el mismo porcentaje de conjugación que el AMP. No atribuimos mucha importancia al resultado obtenido, pues Cohen y McGilvery (2), entre otros autores, han hecho observaciones análogas. Además, hemos podido comprobar recientemente que el sobrenadante libre de microsomas, obtenido a partir del homogeneizado, es capaz de formar fenilsulfato (en anaerobiosis) con el solo agregado de ATP (7).

TABLA 6

Síntesis del p-nitrofenilsulfato por fracciones del homogeneizado de hígado de rata.

Medio: 78 ml ClK 0.154 M, 2 ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M, pH 7; succinato de sodio 0.01 M, AMP 0.001 M, p-nitrofenol 0.9×10^{-4} M. Temperatura 37.5° C; fase gaseosa O_2; incubación 1 hora; 4 ml de homogeneizado al 10 por ciento o fracciones.

Todos los datos representan microgramos de p-nitrofenol combinado.

Exp.	Homogeneizado	Sedimento	Sobrenadante	Sedimento + Sobrenadante	Mitocondrias	Sobren. libre de Mitocondr.	Mitocondrias + Sobrenad. libre de mitoc.
1	23.4	0.5	1.7	21.6	—	—	—
2	35,3	0	0.8	16.5	—	—	—
3	34.7	2.1	—	22	0.6	0.9	25.2

Efecto de la hipotonía y de la congelación. — El medio hipotónico utilizado se obtenía sustituyendo el ClK 0.154 M, presente en el medio usual, por igual volumen de agua destilada. La tabla 5 muestra que en estas condiciones no había formación de p-nitrofenilsulfato. Igual resultado se observaba si se congelaba y descongelaba el homogeneizado en el medio usual, lo que se hizo por tres veces sucesivas.

Fraccionamiento del homogeneizado. — El homogeneizado de hígado al 10 por ciento, obtenido de acuerdo a la técnica antes descripta, se centrifugaba a 600 g durante 15 minutos. El sedimento se volvía a suspender en el medio salino o bien en el sobrenadante obtenido por centrifugación, para ensayar su actividad.

Las mitocondrias se obtenían centrifugando homogeneizado de hígado al 10 por ciento (preparado en sacarosa 0.25 M) durante 5 minutos a 600 g, descartando el sedimento (constituído por células intactas, fragmentos celulares, núcleos y algunos glóbulos rojos) y centrifugando nuevamente el sobrenadante. La centrifugación se efectuaba a 8000 g durante 10 minutos y el sedimento (constituído por mitocondrias) se resuspendía en un volumen de medio salino no fortificado (78 ml ClK 0.154 M, 2 Ml SO_4Mg 0.154 M, 20 ml mezcla reguladora de fosfato de sodio 0.1 M pH 7) igual al volumen original de sobrenadante, centrifugando nuevamente a 8000 g durante 5 minutos. Este sedimento de mitocondrias se suspendía en el medio salino fortificado o en el sobrenadante libre de mitocondrias, para estudiar su actividad. Se utilizó un homogeneizado preparado en sacarosa 0.25 M, porque de acuerdo a Hogeboom y col. ([9]), las mitocondrias se aglutinan en un medio salino y sedimentan más rápidamente, con lo que el rendimiento es bajo y la separación menos neta. El sobrenadante libre de mitocondrias se obtuvo a partir de un homogeneizado (al 10 por ciento), preparado en el medio salino fortificado, centrifugando 5 minutos a 1500 g y luego 10 minutos a 8000 g. El sobrenadante se separaba del sedimento por succión, dejando 1 cm de espesor de fluído sobre el mismo, para evitar contaminación con partículas. No se utilizó sobrenadante preparado en sacarosa 0.25 M, porque la presencia de esta sustancia interfiere en la determinación del p-nitrofenol total. Pudo comprobarse sin embargo, que la formación de p-nitrofenilsulfato parecía producirse en sacarosa 0.25 M, debidamente fortificada, pues los valores obtenidos para el p-nitrofenol libre así lo indicaban. Durante todo el curso del fraccionamiento y antes de la incubación la temperatura del preparado se mantenía entre 0 y 5° C. En la tabla 6 se consignan los resultados obtenidos. Por ellos puede verse que tanto el sedimento como el sobrenadante, obtenidos centrifugando a 600 g durante 15 minutos, muestran muy baja o ninguna actividad cuando se incuban separadamente, pero son activos si se mezclan. Lo mismo se observa en el caso de los mitocondrias y del sobrenadante libre de mitocondrias.

Además de los aspectos antes mencionados se estudiaron algunos otros. Con la idea de que el pirosulfato pudiera ser una etapa intermedia en la formación de p-nitrofenilsulfato cuando se agregaba al preparado que en el medio por pirosulfato de potasio a una concentración final de 0.0015 M. Los resultados fueron todos negativos, comprobándose que no se debían a una acción inhibidora, porque el pirosulfato no impedía la formación de p-nitrofenilsulfato, cuando se agregaba al preparado que contenía AMP y succinato.

Resumen

1. La síntesis de p-nitrofenilsulfato por homogeneizado de hígado de rata (fortificado con succinato 0.01 M y AMP 0.001 M) decrece al aumentar la concentración de p-nitrofenol. Representa un 66 a 78 por ciento, del sustrato agregado, para una concentración de 0.9×10^{-4} M; es del 30

a 35 por ciento para 1.8×10^{-4} M y nula para 3.6×10^{-4} M. Se reduce al 5 por ciento, en el primer caso, en ausencia de sulfato.

2. El fenol (2.7×10^{-4} M) la disminuye en un 20 por ciento.

3. La sustitución del succinato por piruvato, fumarato o citrato no modifica la síntesis de p-nitrofenilsulfato.

4. La metilol gramicidina produce una inhibición del 85 al 98 por ciento.

5. No se observa formación de p-nitrofenilsulfato al sustituir el succinato y AMP por ATP 0.003 M (en anaerobiosis).

6. No hay síntesis en medio hipotónico o después de congelación y descongelación repetidas.

7. El sedimento y el sobrenadante, obtenidos por centrifugación a 600 g durante 15 minutos, así como las mitocondrias y el sobrenadante libre de mitocondrias, no forman p-nitrofenilsulfato si se incuban separadamente, pero lo hacen cuando se mezclan.

8. La sustitución del AMP y succinato por pirosulfato no conduce a un preparado activo.

SUMMARY

1. The synthesis of p-nitrophenylsulfate by rat liver homogenate (fortified with 0.01 M succinate and 0.001 M AMP) decreases with the increase of p-nitrophenol concentration. It is of the order of 66 to 78 per cent, of added substrate, at a concentration of 0.9×10^{-4} M; of 30 to 35 per cent for 1.8×10^{-4} M and no synthesis is observed for a 3.6×10^{-4} M concentration. Without sulfate it drops to 5 per cent (substrate concentration 0.9×10^{-4} M).

2. Phenol (2.7×10^{-4} M) decreases the formation of p-nitrophenylsulfate by 20 per cent.

3. No change is observed if pyruvate, fumarate or citrate are substituted for the succinate.

4. Methylol gramicidin (3×10^{-5} M) produces 85 to 98 per cent inhibition.

5. No formation of p-nitrophenylsulfate (in anaerobiosis) is observed if the succinate and AMP are replaced by 0.003 M ATP.

6. Hypotonicity, and repeated freezing and thawing destroys the activity.

7. The sediment and the supernatant obtained by centrifugation at 600 g for 15 min., and the mitochondria and mitochondrium-free supernatant are individually inactive. The activity is restored when these fractions are combined.

8. Negative results are obtained when AMP and succinate are replaced by pyrosulfate.

Agradecemos la ayuda prestada en este trabajo por la Srta. Ema Fabiani.

BIBLIOGRAFÍA

(1) CLOWES, G. H. A., KELTCH, A. K., STRITTMATER, C. F., WALTERS, C. P.: *J. Gen. Physiol.*, 1950, *33*, 555.
(2) COHEN, P. P., MCGILVERY, R. W.: *J. Biol. Chem.*, 1947, *169*, 119.
(3) CROSS, R. J., TAGGART, J. V., COVO, G. A., GREEN, D. E.: *J. Biol. Chem*, 1949, *177*, 655.
(4) DE MEIO, R. H., TKACZ, LYDIA: *Arch. Biochem.*, 1950 *27*, 242.
(5) DE MEIO, R. H., TKACZ, LYDIA: *J. Biol. Chem.*, 1952, *195*, 175.
(6) DE MEIO, R. H., WIZERKANIUK, MARTHA: Resumés des Communications (II[e] Congrés International de Biochimie), 1952, 306.
(7) DE MEIO, R. H., WIZERKANIUK, MARTHA: Trabajo en preparación.
(8) GARTON, G. A., ROOBINSON, D., WILLIAMS, R. T.: *Biochem. J.*, 1949, *45*, 65.
(9) HOGEBOOM, G. H., SCHNEIDER, W. C., PALLADE, G. E.: *J. Biol. Chem.*, 1948, *172*, 619.
(10) HOTCHKISS, *R. D.* en NORD, F. F. WERKMAN, C. H.: *Gramicidin, tyrocidine, and tyrothricin*: *Advances in enzymology and related subjects*, Interscience Publishers, Inc., New York, 1944, *4*, 153.
(11) HOTCHKISS, R. D. en GREEN, D. E.: *Chemotherapy*: *applied cytochemistry. Currents in biochemical research.* Interscience Publishers, Inc., New York, 1946, 379.
(12) HUGGINS, C., SMITH, D. R.: *J. Biol. Chem.*, 1947, *170*, 391.
(13) MEYERHOF, O., FIALA, S.: *Biochim. et Biophys., Acta*, 1950, *6*, 1.
(14) SOMOGYI, M.: *J. Biol. Chem.*, 1930, *86*, 655.
(15) WILLIAMS, R. T.: *Detoxication mechanisms*, John Wiley & Sons, NewYork, 1949, pág. 136.

METODO PARA LA DETERMINACION CUANTITATIVA DE LA ACTIVIDAD GONADOTROFICA EN LA ORINA *

M. X. ZARROW, W. T. SALTER ** Y E. A. LAZO-WASEM

(Departamento de Ciencias Biológicas. Purdue University, West Lafayette, Indiana y Laboratorios de Farmacología y Toxicología. Yale University School of Medicine, New Haven, Connecticut)

Es de suma importancia el tener información respecto al estado funcional de la hipófisis, cuando se hacen diagnósticos de enfermedades endocrinas que implican el eje pituitario-gonadal. Tal información es de gran valor para determinar si la causa primordial de ciertas afecciones reside en la hipófisis o en las glándulas sexuales. En casos de hipogonadismo, esterilidad y amenorrea, por ejemplo, es de mucha importancia saber si el trastorno es debido a una hipofunción de las glándulas sexuales o a la disminución de la producción de hormonas gonadotróficas por la hipófisis.

Varios procedimientos, ya biológicos, ya químicos, han sido estudiados para la determinación de andrógenos, estrógenos y la progesterona. Tales técnicas de análisis han sido usadas intensamente en laboratorios clínicos y de investigación. En la determinación de hormonas gonadotróficas, sin embargo, hay que tomar en cuenta varios factores que no existen en el caso de los esteroides.

En primer lugar, las hormonas gonadotróficas urinarias son de naturaleza proteica y no hay aún métodos para su determinación. En segundo lugar, las hormonas gonadotróficas existen como complejos de dos o más substancias, cuya purificación y separación de la orina no se ha logrado hacer. Finalmente, dos de los principios hipofisiarios, v. g. la hormona folículo estimulante y la hormona que produce la luteinización del folículo, muestran acciones sinérgicas. No obstante estas dificultades, ha sido posible obtener resultados en este y otros laboratorios. El presente

---

* Esta investigación fué sufragada mediante donaciones del "Office of Naval Research", proyecto N60RI44T.O.VI. del "National Cancer Institute", donación Nº C-290 y de "Schering Corporation, Bloomfield New Jersey".

** El Dr. Dn. W. T. Salter falleció en julio de 1952.

Recibido para su publicación en Noviembre 25 de 1952.

trabajo se refiere a un procedimiento que ha sido estandardizado para uso clínico y de investigación. En este informe se han incluído datos representativos de la excreción gonadotrófica de personas sanas y de pacientes.

MÉTODOS

Extracción de la orina

1. — Se junta la orina fresca de 24 horas en un frasco que contenga 10 cm^3 de ácido acético glacial.

2. — Se añade más ácido acético, si necesario, hasta ajustar el pH a un valor aproximado de 5.

3. — Se filtra y se añade cloruro de sodio hasta alcanzar una concentración de 1 %.

4. — Se añaden 5 volúmenes de alcohol etílico (95 %).

5. — Se deja en la heladera por la noche o hasta que el líquido que sobrenada esté claro.

6. — Se aspira el líquido sobrenadante y se junta el material precipitado en tubos de centrifugación; finalmente el precipitado se debe centrifugar.

7. — Se lava el precipitado con 50 ó 60 cm^3 de éter, quebrándose el precipitado cuidadosamente.

8. — Centrifúguese. Luego se descarta el éter y el precipitado se pone a secar en un desecador.

9. — Se extrae el precipitado tres veces con 25 cm^3 de agua destilada. Después de haber quebrado el precipitado, cada extracción debe durar 30 ó 40 minutos, decantándose el líquido después de cada extracción.

10. — Se combinan los tres líquidos de extracción y se dializa contra agua por 4 horas.

11. — Se reprecipita el dializado con 5 volúmenes de alcohol etílico (95 %) después de haber añadido 0.2 g de cloruro de sodio.

12. — Se deja en la heladera por la noche.

13. — Se aspira la mayor parte del líquido sobrenadante y se junta el precipitado en un tubo de centrifugación de 50 cm^3, de capacidad.

14. — Se lava el precipitado con 20 cm^3 de éter, se centrifuga y se seca en un desecador al vacío. Este precipitado final se puede almacenar a la temperatura ambiente.

Preparación del extracto para inyección

Veinticuatro horas antes de empezar la prueba se añade 6 cm^3 de agua destilada al precipitado y se deja en la heladera.

Antes de inyectar los animales en experimento, se preparan las siguientes diluciones:

Unidades previstas	Volumen del extracto	Volumen del agua	Número de ratones
	cm^3	cm^3	
5	1.20	—	1
10	1.80	1.80	3
20	0.90	2.70	3
40	0.45	3.15	3
80	0.22	3.37	3
160	0.11	3.48	3

Procedimiento para la prueba

1. — Se usan ratones hembras de 21 días de edad.

2. — Cada ratón recibe 0.2 cm^3 del extracto por vía subcutánea de acuerdo con el siguiente horario:

Lunes a las 4 p.m.; martes, miércoles y jueves a las 9 a.m. y a las 4 p.m.

3. — Se matan los animales veinticuatro horas después de la última inyección.

4. — Se disecan los úteros, se cortan por el medio para evacuar el líquido intrauterino, se secan cuidadosamente con papel poroso y se pesan en una balanza de torsión hasta el último décimo de milígramo.

5. — Se preparan curvas de regresión para cada muestra de orina. De estas curvas se puede deducir las unidades de actividad presentes en cada muestra de orina. Una "unidad uterina de ratón" (UUR) se define como la actividad que puede duplicar el peso del útero cuando dicho peso se compara con el de ratones sin tratamiento.

RESULTADOS

Los datos presentados en las Tablas 1 y 2 muestran el contenido gonadotrófico que fué encontrado en la orina de hombres y mujeres sanos. A pesar de que los datos presentados en la Tabla I se limitan únicamente a doce hombres, muestran cierta consistencia. Estos datos muestran que existen valores bajos en los dos extremos de edad y que los valores más altos son los encontrados en la segunda y tercera décadas de vida. Dentro de cada década los resultados son constantes, v. g., los valores para la tercer década varían únicamente de 15 a 25 UUR.

Un total de veinte pruebas fueron hechas en diez mujeres cuya edad se extendía de 16 a 60 años (Tabla 2). Excepto en el caso de mujeres que ya habían pasado la menopausa, se obtuvieron dos muestras de orina. La una se obtuvo durante la fase proliferativa y la otra durante la fase progestacional del ciclo. En algunos casos se hicieron pruebas repetidas de muestras urinarias que se había colectado durante dos ciclos diferentes de la misma persona. Los datos que se presentan en la Tabla 2 muestran que en la mujer la variación es más notoria que en el hombre. Los valores más

TABLA 1

Excreción de Gonadotrofinas en la Orina de Hombres Sanos

Edad (años)	Actividad Gonadotrófica en 24 horas (U.U.R.)	Promedio (U.U.R.)
15-20	12 14 18	15
21-30	15 20 20 20 25	20
31-40	20 25	23
41-50	14 18	16

U.U.R. — Unidad Uterina de Ratón.

TABLA 2

Excreción de Gonadotrofinas en la Orina de Mujeres Sanas

Edad (años)	Actividad Gonadotrófica por 24 horas en UUR		
	Fase Proliferativa	Fase Progestacional	Menopausa y Postmenopausa
16-20	11 12	— 7	
	16; 20 14; 16	8; 10 8; 10	
21-30	25 12 20	12 10	
41-50			80
51-60			180 5

Nota: Los números repetidos se refieren a pruebas hechas sobre diferentes muestras de orina de la misma persona.

bajos en la mujer ocurren durante la segunda década de vida (7 a 12 UUR). Llama la atención el hecho que en una de las mujeres allende de la menopausa se haya encontrado una actividad gonadotrófica muy baja, mientras que en otras dos se obtuvieron los valores altos que son de esperarse. Diferencias consistentes fueron obtenidas durante las dos fases del ciclo menstrual. Un nivel alto de excreción gonadotrófica fué observado durante la fase folicular o proliferativa. Finalmente, se debiese anotar que las pruebas de repetición hechas durante diferentes ciclos menstruales de la misma mujer se mostraron en perfecto acuerdo.

Los estudios sobre la excreción gonadotrófica de un grupo de pacientes con diagnóstico de hipogonadismo, adenoma pituitario, hipopituitarismo y amenorrea (Tabla III), dieron los siguientes resultados. De doce casos de hipogonadismo masculino, cinco casos (aproximadamente 42 %) mostraron una producción gonadotrófica anormalmente baja, dos casos (17 %) moderadamente baja, otros dos casos definitivamente alta y en tres casos (25 %) la producción gonadotrófica se juzgó normal. En un caso de adenoma pituitario y en otro de hipofunción de la misma, ambos hombres mostraron excreciones urinarias indiscutiblemente bajas de gonadotrofinas. En tres de los seis casos de amenorrea estudiados se encontró una excreción muy baja de gonadotrofinas; en un caso se encontraron valores relativamente bajos, en otro caso se encontraron cantidades normales y en un último caso la excreción de gonadotrofina pareció estar anormalmente alta.

Discusión

Varios investigadores han mencionado la excreción urinaria de hormonas gonadotróficas en el hombre y mujer sin embarazo. Tres métodos de extracción han sido usados. Estos son: 1) precipitación por alcohol [10, 13, 14]; 2) precipitación por ácido tánico [11] y 3) usando ultrafiltración [4]. Esta investigación se limita al método de precipitación por medio del alcohol. Por este procedimiento la orina rara vez es suficientemente tóxica para causar la muerte de los animales en experimento.

Varios autores han usado el ratón y la rata como animales de prueba. En nuestra experiencia el ratón es más sensible y además el peso uterino es un índice más sensible que el peso del ovario. Heller, Heller y Severinghaus [8] y también Heller y Nelson [9] han expresado la actividad de la hormona foliculoestimulante en términos del peso ovárico. Klinefelter, Albright y Griswold [10], por el contrario, han usado el diametro del útero del ratón como índice de dicha actividad, habiendo expresado sus valores en términos de mínimo y máximo. McCullagh [12] también expresó sus valores de esta manera. Otros investigadores, por otra parte, han expresado sus resultados en cifras absolutas [1, 2, 3].

Los resultados de este trabajo muestran que es posible obtener una serie contínua de datos de la excreción de gonadotrofinas. Una vez que se establecen curvas de regresión para cada muestra de orina, es posible

obtener directamente la concentración de gonadotrofinas en términos de unidades biológicas. Este procedimiento ha probado ser suficientemente sensible para mostrar una excreción más alta de gonadotrofinas durante la fase proliferativa del ciclo menstrual. Se sabe desde hace algún tiempo que al menos dos principios activos existen en el complejo gonadotrófico y que estos ejercen una acción sinérgica. El efecto de la hormona foliculoestimulante no sólo es grandemente aumentado por la hormona luteinizante ([5, 6]), sino que parece requerir la presencia de ésta última para poder producir algún efecto sobre el útero ([7]). Aparentemente la técnica que aquí se presenta mide un complejo de dos substancias. Sin embargo, los resultados consistentes observados en sujetos normales y los cambios drásticos anotados en casos de hipogonadismo y otras afecciones endocrinas indican que se puede confiar en este método para el diagnóstico clínico.

TABLA III

Excreción de Gonadotrofinas en la Orina de Personas con Enfermedades Endocrinas

Diagnóstico Provisional	Paciente	Sexo	Edad	Actividad Gonadotrófica (UUR en 24 hrs.)
Hipogonadismo	Bro	M	47	5
	Ath	M	25	5
	Hal	M	23	5
	Wig	M		5
	Iss	M	20	7; 6
	Hat	M	36	5
	Ein	M	19	15
	The	M	26	13; 16
	Hil	M	25	9
	Tas	M	17	13
	Tro	M	35	80
	Cha	M	25	125; 110
Adenoma Hipofisario	Jac	M	47	5; 5; 5
Hipopituitarismo	Nap	M	27	5
Amenorrea	Mes	F	20	5
	Fis	F		5
	Lon	F	31	5
	Sai	F	25	7
	Pat	F	32	18
	Man	F	24	140

Nota: Los números repetidos se refieren a pruebas hechas sobre diferentes muestras de orina de la misma persona.

Por medio de este procedimiento se pueden observar los cambios que ocurren con la edad en personas sanas y asimismo los cambios que acaecen en la mujer durante el ciclo menstrual y la menopausia. Además se puede discernir por esta técnica las varias clases de respuesta pituitaria a juzgar por los casos de hipogonadismo presentados aquí. Estas respuestas incluyen producción normal, aumentada o disminuída de gonadotrofinas por la hipófisis. Respecto al caso del adenoma hipofisario se juzgó que había una hipofunción de la hipófisis, ya que la excreción de gonadotrofinas urinarias se mostró muy baja. Los pacientes con amenorrea también se pueden clasificar en tres categorías respecto al funcionamiento hipofisiario, es decir, secreción normal, aumentada y disminuída. Para poder explicar estas reacciones divergentes de la hipófisis, sería necesario un estudio más detallado de los mecanismos implicados.

RESUMEN

1) Se presenta un procedimiento para la determinación de gonadotrofinas en la orina que es aplicable a la rutina clínica.

2) Los valores son expresados en unidades absolutas basadas en métodos biológicos.

3) El método hace uso del útero del ratón y se establecen curvas de regresión para cada muestra de orina.

4) Se define una unidad uterina de ratón (UUR) como la actividad gonadotrófica correspondiente a un volumen de orina que produce duplicación del peso uterino.

5) Se presentan valores correspondientes al nivel de gonadotrofinas en la orina de personas sanas y de pacientes sufriendo de hipogonadismo, adenoma pituitario, hipofunción pituitaria y amenorrea.

6) Se mostraron los cambios de excreción gonadotrófica que ocurren durante el ciclo menstrual.

7) Se concluye que este procedimiento es de valor en el diagnóstico de condiciones patológicas hipofisario-gonadales.

SUMMARY

1) A procedure applicable to routine clinical use for the determination of gonadotrophins in the urine is presented.

2) The values are expressed in absolute units based on a biological end point.

3) The assay uses the uterus of the mouse and regression lines are established for each 24 hours sample of urine.

4) One mouse uterine unit of gonadotrophin is defined as that activity corresponding to a volume of urine that will produce a doubling in the weight of the uterus.

5) Values for the gonadotrophin concentration in the urine of normal men and women are presented, together with values for patients with provisional diagnoses of hypogonadism, pituitary adenoma, hypopituitarism and amenorrhea.

6) In the normal female a consistent pattern in gonadotrophin excretion was noted; the gonadotrophins were higher during the proliferative stage than in the progestational stage of the mentrual cycle.

7) It is concluded that this determination is of definite aid in the diagnosis of pituitary-gonad dyscrasias.

BIBLIOGRAFÍA

(1) CATCHPOLE, H. R., GREULICH, W. W.: The excretion of gonadotrophic hormone by prepuberal and adolescent girls. *J. Clin. Invest.*, 1943, *22*, 799.

(2) CATCHPOLE, H. R., HAMILTON, J. B., HUBERT, G. R.: Gonadotropin excretion. Effect of male hormone therapy on urinary gonadotropins in man. *J. Clin. Endoc.*, 1942, *2*, 181.

(3) D'AMOUR, F. E., FUNK, D., LIVERMAN, H.: Daily gonadotropic hormone tests during 50 complete cycles. *Am. J. Obstet. Gynaec.*, 1940, *37*, 940.

(4) EVANS, H. M., GORBMAN, A.: Urinary gonadotropins in normal men. *Proc. Soc. Exp. Biol. N. Y.*, 1942, *49*, 674.

(5) FEVOLD, H. L.: Synergism of the follicle stimulating and luteinizing hormones in producing estrogen secretion. *Endocrinology*, 1941, *28*, 33.

(6) FEVOLD, H. L., HISAW, F. L., GREEP, R. O.: Comparative action of gonad-stimulating hormones on the ovaries of rats. *Endocrinology*, 1937, *21*, 343.

(7) GREEP, R. O., VAN DYKE, H. B., CHOW, B. F.: Gonadotrophins of the swine pituitary. I. Various biological effects of purified thylakentrin (FSH) and pure metakentrin (ICSH). *Endocrinology*, 1942, *30*, 635.

(8) HELLER, E. J., HELLER, C. G., SEVERINGHAUS, E. L.: Gonadotropic hormone: Assays of human male urine. *Endocrinology*, 1941, *29*, 1.

(9) HELLER, C. J., NELSON, W. O.: Hyalinization of the seminiferous tubules associated with normal or dailing Leydig cell function. *J. Clin. Endoc.*, 1945, *5*, 1.

(10) KLINEFELTER, H. F., ALBRIGHT, F., GRISWOLD, G. G.: Experience with a quantitative test for normal or decreased amounts of follicle stimulating hormone in the urine of endocrinological diagnosis. *J. Clin. Endoc.*, 1943, *3*, 529.

(11) LEVIN, L., TYNDALE, H.H.: Urinary gonadotropin in man. *Proc. Soc. Exp. Biol.*, N. Y., 1935, *34*, 516.

(12) MCCULLAGH, E. P.: Sex hormone deficiencies. Some clinical considerations. *Recent Progress in Hormone Research*, edited by G. Pincus, 1948, *2*, 295.

(13) MCCULLAGH, D. R., BOWMAN, W. E.: The extraction and assay of gonadotropic hormones from human male urine. *Endocrinology*, 1940, *27*, 525.

(14) VARNEY, R. F., KOCH, F. C.: A method for the assay of gonadotropin content of normal human urine. *Endocrinology*, 1942, *30*, 399.

ALTERATIONS OF NEUROMUSCULAR SYNAPSIS DURING WALLERIAN DEGENERATION (*) (**)

C. EYZAGUIRRE, J. ESPÍLDORA AND J. V. LUCO

(Laboratory of Neurophysiology, Catholic University of Chile, Santiago, Chile).

THE MODIFICATIONS which the conduction of nerve impulses undergoes after severance of the axone from the neural soma have been studied repeatedly. Changes in conduction velocity and in the propagated potentials have been related to anatomical alterations produced by Wallerian degeneration (for references see Gutmann and Holubár, 1950). The reaction of the post-neural element during degeneration of motor nerves also has been investigated. Titeca (1935) demonstrated that neuromuscular transmission fails before the nerve has ceased to conduct and subsequently, Lissák, Dempsey and Rosenblueth (1939) have shown that this independent impairement of neuromuscular transmission is a graded phenomenon.

In view of the importance of the end-plate potential on neuromuscular transmission it was thought interesting to study the evolution of this electrical response during the process of Wallerian degeneration. The results obtained led us to consider the influence of the length of the axone, measured from the site of nerve section to the corresponding end-plate, on the evolution of synaptic alterations.

METHODS

Unselected adult cats anaesthetized with sodium pentobarbital (0.6 g per kg) given intraperitoneally were used throughout the 40 experiments performed. The experimental object was a striated muscle, the tenuissimus or abductor cruris. The animal was placed lying on one side so that the external lateral portion of the hind limb looked upwards. The skin was incised from the pelvic bones up to the knee, and the edges of the skin held upwards by means of steel hooks and rubber bands; the cup

(*) Aided by grants for research from The Rockefeller Foundation, New York, and the Fundación Gildemeister, Santiago, Chile.

(**) Read at the "Symposium sobre Neurona y Sinapsis" held at Montevideo, October 1952.

Received for publication December 2, 1952.

thus formed was filled with mineral oil kept at 37°C by an infrared bulb. The biceps was extirpated and the tenuissimus was dissected starting from its pelvic end. The sciatic nerve was acutely sectioned both centrally and peripherally to the emergence, from the main trunk, of a fine nerve twig innervating the muscle under study. This twig was carefully separated from underlying structures and gently placed on two platinum hooks which served as stimulating electrodes. Stimuli were always slightly supramaximal square pulses delivered by a Grass Stimulator. The stimulus duration was 20 microseconds. The muscle was freed in an extension which ranged from 8 to 10 cm, special care being taken to preserve its blood supply intact. Occasionally the muscle was fixed at both the pelvic and tibial ends by means of steel needles which could be displaced along the longitudinal axis of the muscle, to give an appropriate tension in order to obtain isometric contractions. The recording electrodes consisted of two platinum wire hooks over which the tenuissimus was gently placed. They were mounted on ball and socket joints attached to calibrated micrometer screws. This arrangement allowed a rectilinear travel along all the axis of the muscle. The preparation was immersed in the oil. Ditopic pick-up electrodes were always used, separated by an interval of 2 mm, distance which was maintained constant during all experimental series. Records were taken millimeter by millimeter along the length of the muscle. Action potentials were led through capacity-coupled Grass preamplifiers to a R. C. Cossor Double Beam Oscillograph and photographed. In order to detect end-plate potentials the preparation was curarized with d-tubocurarine injected into the abdominal aorta. To study the effects of Wallerian degeneration, the sciatic nerve was aseptically sectioned from 24 to 73 hours before the experiment. The section was performed from 2 to 3 cm centrally to the emergence of the twig innervating the muscle. In order to simplify experimental conditions the portion of muscle from the nerve entry toward the pelvic end was not considered in these experiments. The locus at the nerve entry was labeled locus O and the other loci were numbered accordingly with the distance in millimeters from the nerve entry.

RESULTS

A) *The electric pattern of a normal tenuissimus.* — It was necessary to know the electric pattern of a normal muscle before studying the effect of Wallerian degeneration on the activity of this muscle. The normal tenuissimus was explored along its longitudinal axis from 5 to 8 cm, during the indirect stimulation produced by supramaximal pulses delivered at a frequency of 1.5 per second. A typical observation is shown in fig. 1. As it is well known the dipolar pick-up electrodes recorded polyphasic potentials. The majority of the responses were triphasic although biphasic and tetraphasic potentials also were observed.

The fact that a shift in phase of the propagated potential corresponds to a zone with high concentration of end-plates (Jarcho, Eyzaguirre, Berman and Lilienthal, 1952), was confirmed in this study: under curare the end-plate potentials of highest voltage were found at the loci of phase

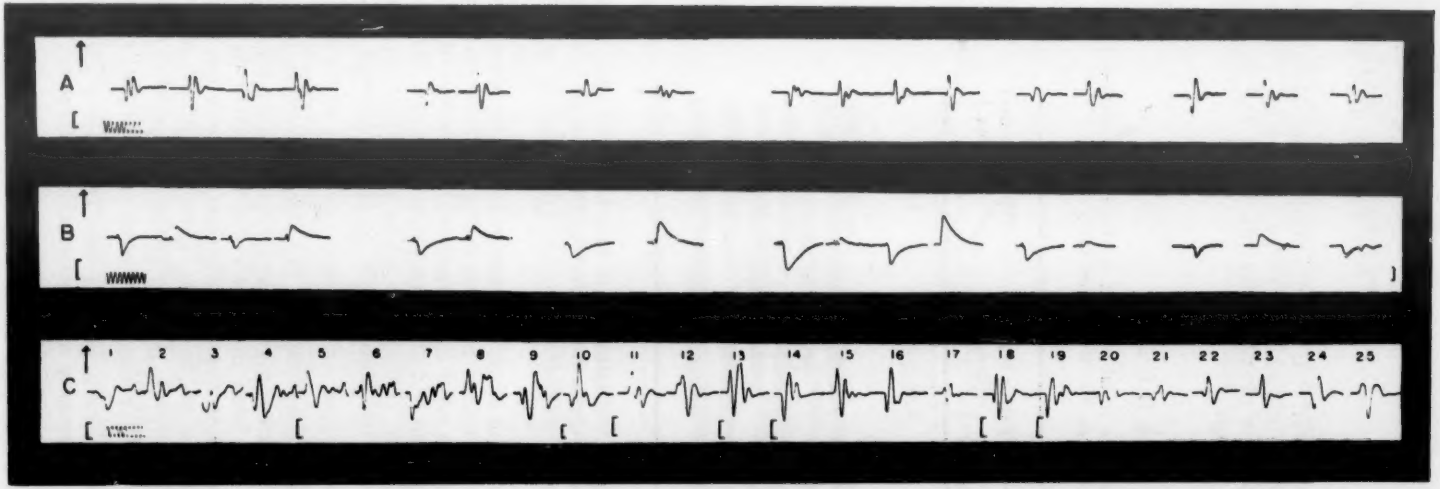

FIG. 1. — Each one of the white rectangles is a schematic representation of the tenuissimus. Arrows indicate the nerve entry. Abscissae A and B are the same. A: Normal electromyogram. Ditopic recording (inter-electrodal distance: 2 mm). Records were taken at millimeter intervals. Only some of the responses are illustrated. In this figure the position of the electric potential corresponds to the locus where they were recorded. In A and B 5.5 cm of muscle were explored. Cal: 5 mV.

B: The same experiment as in A but after the administration of d-tubocurarine. Cal: 0.1 mV. Tho last two records must be measured accordingly with calibration of the right: 0.2 mV.

C: Electromyogram of a muscle in which the nerve was sectioned 49 hours before the experiment. Method similar to A. The distance between the recorded responses is 3 mm (7.5 cm of muscle were explored). The numbers only indicate a reference for calibration. Calibrations: from 1 to4: 0.1 mV (1); from 5 to 9: 0.2 mV (5); of 10: 0.2 mV; from 11 to 12: 0.5 mV (11); of 13: 0.5 mV; from 14 to 17: 1 mV (14); of 18: 1 mV; from 19 to 25: 2 mV (19). Time: 1 msec.

shifting. The results demonstrated that the end-plate concentration zones were disposed following the longitudinal axis every 4 or 5 mm. The number of end-plates in each zone is probably not the same: the potentials observed were not of equal voltage.

TABLE I

Voltage of propagated potentials. The tibial segment is about 5 cm distant from the pelvic segment. For details see text.

Time of degeneration Hours	Pelvic segment millivolts	Tibial segment millivolts
0	5.42 ± 0.33	4.24 ± 0.31
48	0.12 ± 0.01	1.54 ± 0.1
66	0.0	0.32 ± 0.04
73	0.0	0.16 ± 0.01

Although the propagated potentials did not have similar voltage values (fig. 1-A), the average values were practically constant. To calculate this average, voltages were measured at every millimeter in segments of 10 mm (Table 1).

A diagram summarizing the electrical responses of the muscle both in normal and curarized preparations is given in fig. 2. The upper part shows the muscle and the nerve with its intramuscular branching. Each branching would correspond to an end-plate concentration zone. The lower part has been taken from Adrian (1925) ; the white dots which have been added correspond to the electrical localization of the end-plates within the fibers.

B) *Effect of Wallerian degeneration on the electric pattern of muscle.* — After having studied normal muscles experiments were performed in which the sciatic nerve had been previously cut (see Methods). The alteration of synaptic transmission due to Wallerian degeneration started at 24 to 48 hours after severance of the nerve. These alterations progressed until the nerve ceased to conduct. The following four aspects characterize the evolution of the alterations:

(a) *low voltage potentials:* as it may be seen in Table 1 the height of the propagated responses decreased as degeneration progressed. There

is a clear cut difference between the voltage values of the normal potentials and those of propagated responses obtained during Wallerian degeneration. At a given time this decrease in voltage was more marked in the pelvic segment than in the tibial segment.

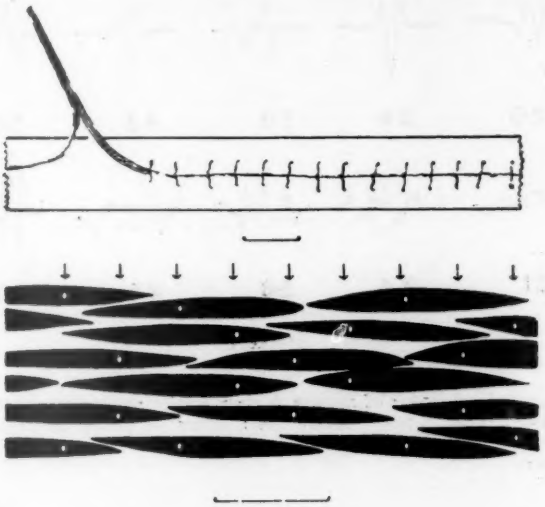

FIG. 2. — Schematic diagram of the tenuissimus. *Upper part*: the rectangle represents a portion of the muscle; see also nerve entry and intramuscular branching of the nerve. *Lower part*: distribution of the muscle fibers. The white dots indicate a probable localization of end-plates; arrows indicate end-plate concentration zones. Calibration: 1 cm.

(b) *polyphasic potentials:* the propagated response showed a tendency to become more polyphasic. In some instances potentials having up to nine phases were recorded. Sometimes it was not possible to record a clear cut spike potential because the propagated responses consisted of several slow deflections of rather uniform voltage. In fig. 3 the difference between normal propagated responses and those obtained 66 hours after the section of the nerve appears evident.

(c) *traces of end-plate potentials:* as is well known the end-plate potential appears when the preparation has been either fatigued or curarized. Our experiments gave evidence that when the nerve suffered Wallerian degeneration some responses were undoubtedly end-plate potentials. These potentials were distorted because propagation was still present.

(d) *clear cut end-plate potentials:* when degeneration lasted longer the chances of recording less distorted end-plate potentials became greater because the voltage of the propagated responses decreased. Also the voltage values of the end-plate potentials dimished progressively. Fig. 4

FIG. 3. — Propagated responses of muscle during Wallerian degeneration. *Upper row*: Normal muscle. Cal: 5 mV. *Lower row*: After 66 hours of degeneration. The numbers indicate the distance in millimeters from the nerve entry. Cal: 0.1 mV. Time: 1 msec.

FIG. 4. — End-plate potentials during Wallerian degeneration. A: Normal muscle. B: Responses obtained after 49 hours of degeneration. C: Responses obtained after 60 hours of degeneration. Numbers indicate the distance in millimeters from the nerve entry. Time: 1 msec.

(points 2 and 36) shows the typical inflection of the potential which is characteristic of a partially curarized or fatigued muscle. Bacq and Coppée (1941) have reported similar observations in frog muscle innervated by a nerve undergoing Wallerian degeneration. As in curarized muscle this inflection is especially noticeable when sub-maximal stimuli are applied to the nerve. This fact is illustrated in fig. 5 where muscle action potentials after single nerve shocks were obtained. The nerve had been cut 66.5 hours previously and was being stimulated with shocks of decreasing intensity.

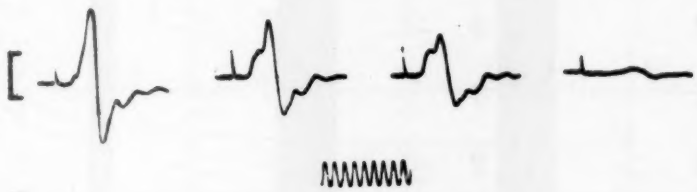

FIG. 5. — End-plate potentials revealed by the use of sub-maximal stimulation. Electric potentials were obtained 66.5 hours after severance of the nerve. All the responses were recorder in a zone where end-plate concentrations had been previously localized. The first potential was produced by maximal stimulation and the others by progressively decreasing voltages. Cal: 0.2 mV. Time: 1 msec.

When the time of degeneration is kept constant and the altered electrical activity is recorded along the longitudinal axis of the muscle, it may be easily noticeable that alterations of the electrical responses started at the locus nearest the nerve entry (locus O, see Methods). These alterations progressed slowly toward the tibial end (figs. 1-C and 6). Accordingly, in an early stage of degeneration, responses of the pelvic segment remained practically normal (fig. 1-C). This phenomenon was not only evident as far as electrical responses were concerned, but sometimes muscular contractions were present only on the tibial segment. In some experiments performed during a later stage of Wallerian degeneration, the number of phases became normal probably due to the decreased voltage of the electrical activity and the disappearence of low voltage phases.

C) *Neuromuscular fatigue.* — The observations reported above suggested the necessity of using higher frequencies of stimulation. The response of normal muscles and that of muscles in which the nerve had undergone Wallerian degeneration was studied using frequencies of from 1 to 100 per second. The activity of both the pelvic and the tibial segments were recorded simultaneously and on certain occasions nerve action potentials were also recorded from the innervating twig (fig. 7).

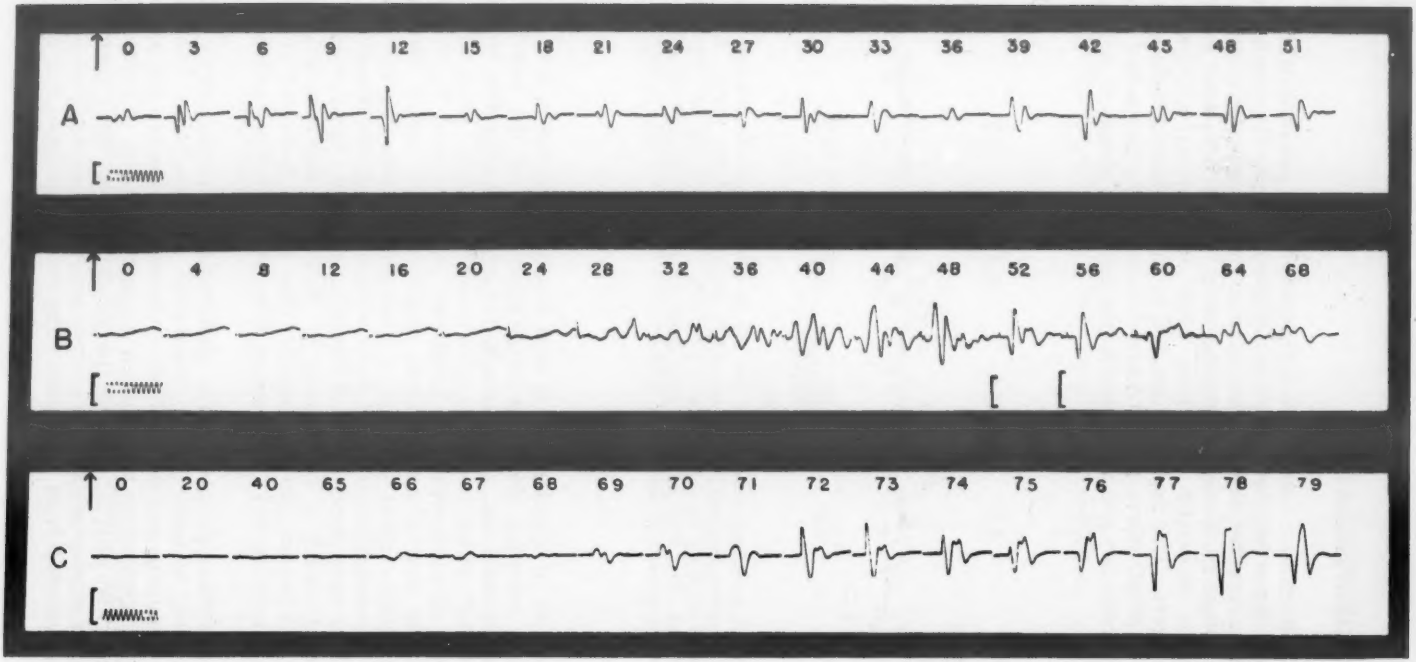

FIG. 6. — Different stages in the course of Wallerian degeneration. For details, see fig. 1. A: Normal muscle. Cal: 5 mV. B: After 66 hours of degeneration. Calibration: from 0 to 48: 0.1 mV. (0); of 52: 0.2 mV.; from 56 to 68: 0.5 mV. (56). C: After 73 hours of degeneration. Cal: 0.2 mV. The numbers indicate the distance in millimeters from the nerve entry. Time: 1 msec.

Fig. 8 illustrates the evolution or depression of the magnitude of the end-plate potentials of a normal tenuissimus stimulated indirectly at a frequency of 30 per second. Previously this preparation had been partially fatigued. Simultaneous recordings of both segments showed that the course of depression of these potentials was practically similar. The propagated responses also showed a similar course. Nevertheless, when the nerve had been cut 60 hours previously the behaviour of the pelvic segment differed from that of the tibial segment (fig. 9). Fatigue ap-

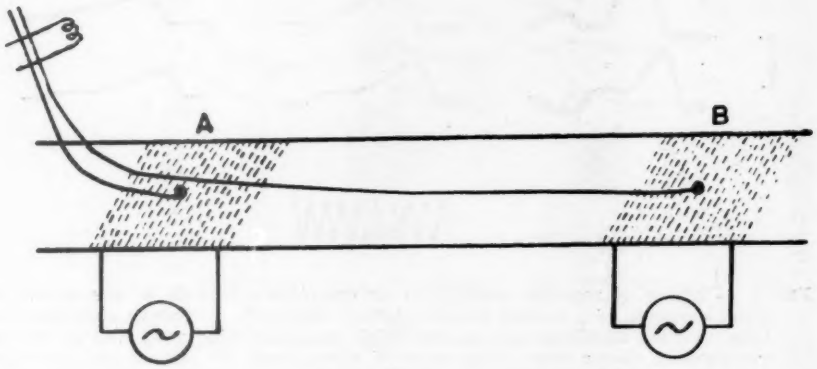

FIG. 7. — Diagram showing the simultaneous recording of two segments. A: Pelvic Segment. B: Tibial Segment.

peared first on the pelvic segment. In the same figure it may be seen that during the process of synaptic fatigue the height of the nerve spike remains unaltered. The curves presented in fig. 10 are the graphical expression of the magnitude of both the propagated responses and the end-plate potentials of either segment. Normal muscles are represented by curve A, and it may be seen that the course of the depression of these potentials from both the tibial and the pelvic segment follows a similar time relation. The mean values, the number of observations and statistical dispersion also are given in fig. 10. A typical experiment of a muscle tenuissimus in which the nerve was sectioned 60 hours before was chosen. This muscle was stimulated indirectly at the same frequency (30 per second) and fatigability of both segments was recorded. It is clear that the pelvic segment (curve C) was fatigued earlier than the tibial segment (curve B).

DISCUSSION

A) *Methods.* — The normal activity of the muscle for periods longer than 10 hours proves that the muscle remains in good physiological condi-

tions during the whole experimental procedure. This preparation —tenuissimus muscle— presents several advantages over similar ones: it is very thin (1 mm) and its width never exceeds 4 mm. Usually it ranged from 2 to 4 mm. Its length is about 10 to 12 cm. All these characteristics make this muscle similar to a nerve in the sense that the electric responses recorded at a given locus result from the activity of a zone covering its whole cross section.

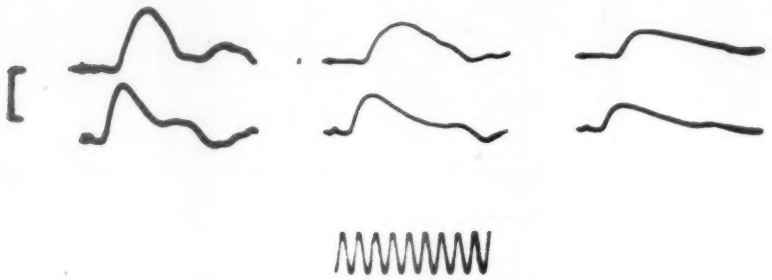

FIG. 8. — Effect of repeated stimulation on end-plate potentials of the pelvic and tibial segments of a normal muscle partially fatigued. End-plate potentials have been recorded simultaneously on the tibial segment (upper row) and on the pelvic segment (lower row). Frequency of stimulation: 30 per second. From left to right: potentials recorded after 10, 30 and 60 seconds of stimulation. Cal: 2 mV. Time: 1 msec.

B) *Normally innervated muscles.* — The electric model of the muscle in fig. 2 was drawn accordingly with the following two facts: (a) the end-plates are grouped at distances of 4 to 5 mm (fig. 1); (b) each fiber is about 2 cm in length (Adrian, 1925). This model explains the presence of polyphasic potentials of different voltage recorded along the muscle. According to Jarcho, Eyzaguirre, Berman and Lilienthal (1952), the record taken at a given locus would be the resultant of responses originated either at the right or left of the electrode, or produced on the very zone of recording (loci marked by arrows in the diagram of fig. 2). This would explain also the shift in phase of the propagated responses on the sites of end-plate concentration. Also the different voltage values are due to the fact that the electrodes are recording simultaneously signals of opposite signs from the left and from the right. Also potential differences may not be obtained at all if there is perfect isochronism and the responses are of equal intensity starting at both sides of the electrode.

C) *Muscles innervated by degenerating nerves.*

1. — *Phenomena recorded at a given locus.*

(a) *Nerve action potentials:* It is well known (Rosenblueth and Dempsey, 1939; Erlanger and Schoepfle, 1946; Gutmann and Holubár,

1950) that a nerve under Wallerian degeneration conducts impulses up to 70 or 80 hours after its severance, that conduction velocity continues showing approximately normal values; and that the nerve ceases to conduct rather abruptly. Also, excitability remains almost normal.

The experiments reported here refer to a situation in which the nerve still conducts, i.e., before the abrupt cessation of conduction, as demons-

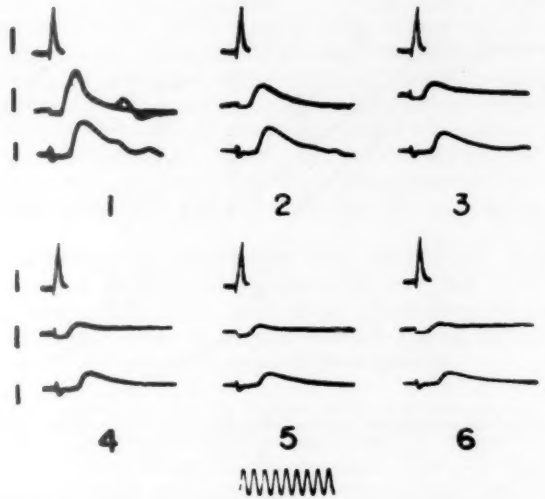

FIG. 9. — Effect of repeated nerve stimulation on end-plate potentials of the pelvic and tibial segments of a muscle partially fatigued after 60 hours of Wallerian degeneration. End-plate potentials were recorded as indicated in fig. 7. In each column, from the top down, the nerve action potentials and the end-plate potential of the pelvic and the tibial segments are represented. From 1 to 6, responses obtained after 5, 15, 30, 45, 65 and 80 seconds of stimulation at a frequency of 30 per second. Cal: Nerve: 0.1 mV. Pelvic segment: 0.1 mV. Tibial segment: 0.2 mV. Time: 1 msec.

trated by the following: (i) the time that elapses between the section of the nerve and stimulation and recording corresponds to a period when the nerve still conducts; (ii) the recording of end-plate potentials is an evident sign that nerve impulses reach the nerve endings; (iii) nerve action potentials remained undisturbed during the entire experiment (fig. 9).

Before the nerve ceases to conduct the first important alteration is a progressive decrease of the muscle spike potential which starts 2 hours after the section of the nerve. Up to that point the nerve spike presents normal values and even values above normal (Lissák, Dempsey and Rosenblueth, 1939). The experiments presented in this study show that after 48 hours of degeneration important alterations of the synaptic me-

chanisms take place. It is obvious then that these alterations can not be related to the height of the nerve spike. Lissák et al. (1939) reported that 48 to 60 hours after the section of the nerve there are certain alterations of the stages of neuromuscular transmission, especially of the 5th stage (Rosenblueth and Luco, 1939) without any disturbance of the nerve action potentials taking place. The present work confirms the observa-

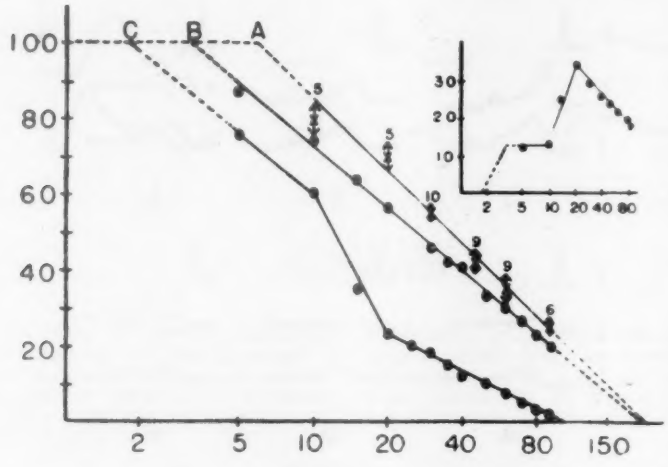

FIG. 10. — Comparative fatigability of the pelvic and tibial segments. Abscissae: Time in seconds. Ordinates: Percentage of height of the response. Curve A: Fatigability of any one segment of a normal muscle. The figures indicate the number of experiments and the arrows ± 2 Σ. Curve B: Fatigability of the tibial segment of a muscle in which the nerve has been degenerating for 60 hours. Curve C: Fatigability of the pelvic segment of the same muscle. Insert: Ordinates: Percentage difference between curves B and C. Abscissae: Time in seconds.

tion that synaptic alterations during Wallerian degeneration start before any abnormality on the nerve spike can be detected.

b) *Muscle action potentials:* One of the first modifications that were recorded was an increased number of phases at a given locus of the muscle. This phenomenon indicates a temporal dispersion of the electric responses of the muscle. A similar result has been described by Hodes, Larrabee and German (1948) and by Jasper and Ballem (1949), although these authors worked under different conditions. They have demonstrated that during re-innervation of a muscle the first potentials that could be recorded were more polyphasic compared to the responses of normal muscles. Once re-innervation is about to be completed the number of phases decreases progressively with a tendency toward normality.

Temporal dispersion can be due to several factors which cannot be elucidated with the present experimental findings. Obviously dispersion indicates that at a given time alterations do not occur in the different recorded elements with the same intensity. Furthermore, this momentary disturbance might be localized: (i) in the mechanisms from which the end-plate potential originates; (ii) in the start of the propagated muscle potential; (iii) in the conduction velocity of the propagated responses in the muscle. As expressed above, our experimental findings do not allow the exclusion of any of these factors or to determine which is the most important one. However, as it may be seen below, not all the synaptic alterations followed an isochronous course. Moreover, the fact that in some experiments an inflection of the curve of the muscle action potentials was found, especially when sub-maximal stimuli were employed (fig. 5), would indicate that the earliest alteration takes place during the generation of the end-plate potential. As it happens during partial curarization. These deflections seem to show that the voltage reached by the end-plate potentials is lower than normal and therefore the propagated potential is delayed.

It seems important to emphasize the paralellism existing between phenomena recorded during curarization, fatigue and Wallerian degeneration. In all these conditions there is a diminution of voltage height and sufficient synaptic delay to render possible a clear distinction between the end-plate potential and the propagated response.

2. — *Phenomena recorded at different loci of the muscle:* Up to now we have discussed the neuromuscular disturbances produced by Wallerian degeneration at a given locus of the muscle. Now we shall discuss the different alterations that occur along the longitudinal axis of the muscle. As expressed in the section "Results", there is a clear difference as to the time when modifications during Wallerian degeneration occur in a portion of muscle close to the nerve section and in a more distant portion. Thus all the observed phenomena —increased number of phases, lower voltage potentials, traces of end-plate potentials, etc.,— appeared first and were more intense, at a given time of degeneration, in regions closer to the sections of the nerve. This fact was especially analyzed in an experimental series in which fatigability of two different segments was studied; one close to the nerve section, innervated therefore by a short nerve, which has been called pelvic segment; and the other, the tibial segment, which is innervated by a long nerve. In fig. 10 it may be seen that fatigability is the same in both segments of a normal muscle. Nevertheless, the process of Wallerian degeneration produces a marked difference in the fatigability of both segments. Potentials recorded on the pelvic segment showed an earlier and more intense fatigue than those of the tibial segment.

When any other alteration produced by Wallerian degeneration is considered, a spatial relation may be observed, that is: modifications progress toward the tibial end of the muscle.

If we consider that both pairs of recording electrodes were separated by a distance of 4 to 6 cm and that the muscle fibers have a length of about 2 cm (Adrian, 1925) it may be concluded that in this experimental

series two different groups of fibers were recorded. Actually there are two different neuromuscular preparations linked by muscle fibers, the response of which did not alter the electric response that was being recorded. Furthermore, since this muscle is so uniform and regular, one may accept that all neuromuscular units have similar functional qualities. How then could one explain the different behaviour of the synaptic mechanisms in these two preparations during Wallerian degeneration? The two preparations differ only in one aspect: the tibial segment is innervated by a nerve which is longer than that innervating the pelvic segment. The distance between the stimulating electrode and the pelvic segment is about 2 cm. whilst the distance between the stimulating electrode and the tibial segment ranges from 6 to 7 cm. Between the site of section and the stimulating electrode there is a distance of about 3 cm. It seems fair to conclude that the only difference is the axone length. This conclusion is followed by a corollary: the axonic influence on the synaptic mechanisms not only depends on its terminal processes, but also on the function of the axone quite far from the synapse. The latter function is modified prematurely during Wallerian degeneration.

For a better understanding of this problem, a discussion of the results under the postulates of the chemical theory of the transmission of nerve impulses will be attempted. Acetylcholine is necessary to activate the end-plate and thus to originate the end-plate potential. Acetylcholine is metabolized in the motoneurone and the stimulus which reaches the nerve endings produces its liberation. Therefore two functions seem to be necessary for the transmitting mechanism: the arrival of the propagated potential at the nerve endings and the liberation of a substance previously metabolized by the nerve. Furthermore it seems legitimate to accept (see Rosenblueth, 1950) that in Wallerian degeneration the timing conditions are different for the liberation of a given quantum of aceytlcholine and for the conduction of nerve impulses. The experiments reported in this paper suggest that the influence of the nerve on the synapse that is mediated by aceytlcholine not only depends on the nerve endings but on the neurone as a whole. This assumption may explain why a longer axone means greater resistance to the consequences of Wallerian degeneration.

Thus there would be two groups of functions whose timing conditions during Wallerian degeneration are different: (i) the conduction of propagated impulses along the nerve depending on the function of the membrane, and (ii) a group of metabolic functions depending on the integrity of the axone. With regard to the first group, three explanations have been given: (a) degeneration follows a centrifugal course (Rosenblueth and Dempsey, 1939); (b) disturbances are distributed at random loci but are more frequent toward the periphery (Erlanger and Schoepfle, 1946); and (c) conduction ceases at any segment of the nerve (Gutmann and Holubár, 1950). The same reasoning may be applied also to the second group of functions: (a) a centrifugal course of the alterations could explain the different behaviour of different loci because the region closer to the nerve lesion would be altered sooner; (b) the second explanation would not be in accordance with these results because the statistical probability of alteration would be higher in the long nerve than in the short

one; (c) the third consideration referred to in the preceding paragraph would not explain the results described in this paper, because it does not consider as an important variable the length of the nerve. Undoubtedly, there still remains the possibility that synaptic alterations would be only the result of metabolic disturbances occuring simultaneously along the whole axone.

SUMMARY

The electric potentials of the tenuissimus muscle of the cat were recorded with the purpose of studying the alterations that the neuromuscular transmission suffers during Wallerian degeneration.

In the first experimental series both normal and curarized muscles were studied (figs. 1 and 2).

In a second experimental series muscles innervated by nerves undergoing Wallerian degeneration were used and the following disturbances were found: (a) decreased voltage of the muscle potentials (fig. 1-C) and an increase in the number of phases of these potentials after a single stimulus (fig. 3); (b) traces of end-plate potentials rather distorted by propagated responses (fig. 4-B); (c) clear cut end-plate potentials (fig. 4-C).

At a given locus of the muscle the alterations follow the order indicated.

A comparison of different segments of the muscle showed that these disturbances progressed from the loci closer to the section of the nerve toward the tibial end of the muscle

In a third experimental series neuromuscular fatigue was studied by measuring the height of both the end-plate potential and the propagated responses. Segments closer to the section of the nerve underwent fatigue earlier than those near the tibial insertion.

The possibility that zones of the axone far apart from the synapse would exert an influence on the synapse is discussed.

REFERENCES

(1) ADRIAN, E. D.: *J. Physiol.*, 1925, *60*, 301.
(2) BACQ, Z. M., COPPÉE, G.: *Acta Biol. Belg.*, 1941, *1*, 67.
(3) ERLANGER, J., SCHOEPFLE, G. H.: *Amer. J. Physiol.*, 1946. *147*, 550.
(4) GUTMANN, E., HOLUBÁR, J.: *J. Neurol. Neurosurg. and Psychiat*, 1950, *13*, 89.
(5) HODES, A., LARRABEE, M. G., GERMAN, W.: *Arch. Neurol. and Psychiat*, 1948, *60*, 340.
(6) JARCHO, L. W., EYZAGUIRRE, C., BERMAN, B., LILIENTHAL, J. L.: *Amer. J. Physiol.*, 1952, *168*, 446.
(7) JASPER, H., BALLEM, G.: *J. Neurophysiol.*, 1949, *12*, 231.
(8) LISSÁK, K. E., DEMPSEY, E. W., ROSENBLUETH, A.: *Amer. J. Physiol.*, 1939, *128*, 45.
(9) ROSENBLUETH, A.: *The Transmission of Nerve Impulses at Neuroeffector Junctions and Peripheral Synapses.* New York, Wiley & Sons., 1950.
(10) ROSENBLUETH, A., DEMPSEY, E. W.: *Amer. J. Physiol.*, 1939, *128*, 19.
(11) ROSENBLUETH, A., LUCO, J. V.: *Amer. J. Physiol.*, 1939, *126*, 39.
(12) TITECA, J.: *Arch. Int. Physiol.*, 1935, *41*, 1.

FATTY DEGENERATION OF THE LIVER CAUSED BY HIGH ALTITUDE HYPOXIA IN YOUNG ALBINO RATS

HUGO CHIODI* AND RODOLFO SAMMARTINO**

(Instituto de Biología de la Altura, Universidad Nacional de Tucumán, Mina Aguilar, Pcia. de Jujuy, Argentina)

ALTHOUGH numerous works have been published on the action of the decrease of barometric pressure or of the concentration of oxygen in the inspired air on the fertility of different species, little attention has been paid to the high death rate and to the difficulty of reaching adult age in animals born under low oxygen pressure.

Considering that in most of cases the animals have been exposed to discontinuous severe hypoxias ([1, 2, 3, 4, 5, 6, 7, 8] and many others) the results cannot be compared to those presented in this paper. We will limit the bibliographical comment to those studies similar to ours as to the continuity and severity of the hypoxia.

Sundstroem and Michaelis ([9]) concluded that rats exposed to a continuous simulated altitude of 5 200 m did not mate or procreate.

According to Accame ([10]), Monge tried to start a colony of white rats in Morococha (4 540 m) and found a complete cessation of reproduction.

Moore and Price ([11]) studied the behaviour of albino rats at 180, 2 250, 2 880 and 4 278 m above sea level during 90 days (only 60 days at 4 278 m) and reached the following conclusions: the body weight and the weight of testicles, prostate gland, seminal vesicles, ovaries, uterus, hypophisis, thyroids, adrenals, heart, lungs, spleen, kidney and liver remained within normal limits. Growth from weaning to sexual maturity was similar at all levels. Sexual maturity appeared at normal age. Reproduction was normal in adults mated 1,30 and 60 days after arriving at different altitudes. The average litter size and the average body weight of the new-

* The facilities granted to the Institute and its staff by the Compañía Minera Aguilar are gratefully acknowledged.

** Chief of the Laboratory of the Chair of Gynecology, Buenos Aires.

Received for publication December 10, 1952.

born showed no change in relation to the altitude. The only abnormality observed at 4 278 m was a decrease in the survival and in the growth rate of young from birth to weaning age, (only 16 out of 87 newborn lived more than 30 days). According to these authors the high mortality is due to the canibalism of the mothers and to a faulty lactation. They relied upon the growth of the suckling young to denote lactation perfomance.

We must point out that Altland and Allen ([5]) observed 70 per cent mortality in the first 21 days among 385 albino rats exposed 4 hours daily since birth, to a simulated altitude of 7 500 m.

The settling of a rat colony in the Instituto de Biología de la Altura, in Mina Aguilar, 3 990 m above sea level, involved the control of the fertility and fecundity as well as of the development and growth rate of the newborn rats, whose high death rate called at once our attention. The investigation of its causes is the object of this paper.

Materials and Methods

To start the rat colony in the Institute's laboratories, a group of adult albino rats, obtained from the homogenous stock of rats of the Instituto de Biología y Medicina Experimental of Buenos Aires, were taken to Mina Aguilar (3 990 m; annual average of barometric pressure: 476 mm Hg). The animals were placed in cement cages ($40 \times 40 \times 40$ cm) the floors of which were covered with wood shavings. The room temperature was constantly kept within 20° and 23° C, with 70-80 per cent of humidity. Rats were fed with:

Milk prepared with powder milk ..	64	per cent
Wheat bread	20	„ „
Corn	8	„ „
Wheat	8	„ „
Brewers yeast	0.2	„ „
Cod liver oil	0.08	.. ..

After mating, each pregnant female was caged separately and all cages were checked daily.

Animals were weighed every other day in a scale sensitive to the g. During the first month of life, that is, while the suckling young were with their mothers the total litter weight was recorded and the individual weight was calculated as an average. Sex was not established until young were a month old.

For the histological study young were killed under ether and the organs fixed in 10 per cent formalin or alcohol 96°.

For hyperpressure experiments, a metalic chamber ($20 \times 30 \times 30$ cm), with glass windows, through which air circulated at a rate of 5 liters per minute was used; this ventilation rate kept the CO_2 concentration below 0.4 per cent. The pressure and air circulation within the chamber were maintained by a compressor and a tank equipped with an automatic

pressure regulator. Air was aspired from the room where the rest of the rats were kept. Pressure inside the chamber oscillated between 0.6 and 0.7 atmospheres above the outside pressure; thus, the rats were under a total pressure of 930 to 960 mm Hg with a partial oxygen pressure of 196-200 mm Hg, that is slightly greater than at sea level. Except for a daily period of 30 minutes for chamber cleaning and food replacement, the pressure was maintained continuously. The chamber was placed in the breeding room to prevent any ambiental change.

RESULTS

In 8 months, 46 rats were born, distributed in 70 litters, of which only 7 survived over 30 days. Of the 7 surviving young, one died at 32, two at

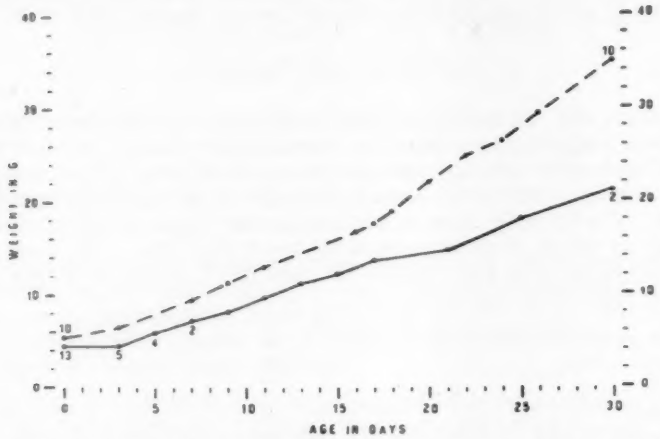

FIG. 1. — *Growth curves of the young rats of two litters born at Mina Aguilar, 3 990 m above sea level.*
—— kept at barometric pressure, annual average 476 mm Hg.
- - - - kept under sea level pressure (hyperpressure chamber), since birth.
Numbers on the lines indicate litter size.

38, one at 42, one at 45 and one at 100 days of age. The remaining was a female who reached sexual maturity and was still alive some months later. She procreated normally but her offsprings died before they were 30 days old.

The mothers ate their dead young more frequently than at sea level, but we never found that they killed them.

Table 1 shows the age distribution of the deaths which occurred in the first 30 days of life of 436 rats born at 3 900. As in most cases it was imposible to establish exactly whether the animals were born dead or died within the first hours following delivery, one group was made of both, being therefore the larger one, which represents 20 per cent of the total.

TABLE 1

Age distribution of deaths among 436 suckling rats born at Mina Aguilar, 3290 m above sea level.

Age in days	0–1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Number of deaths	88	41	24	15	7	6	17	8	9	18	19	17	26	36	13	14

Age in days	17	18	19	20	21	22	23	24	25–30	31–45	100	surviving
Number of deaths	23	4	6	13	7	8	0	7	3	5	1	1

* Includes deadborn and those who died during the first day of life.

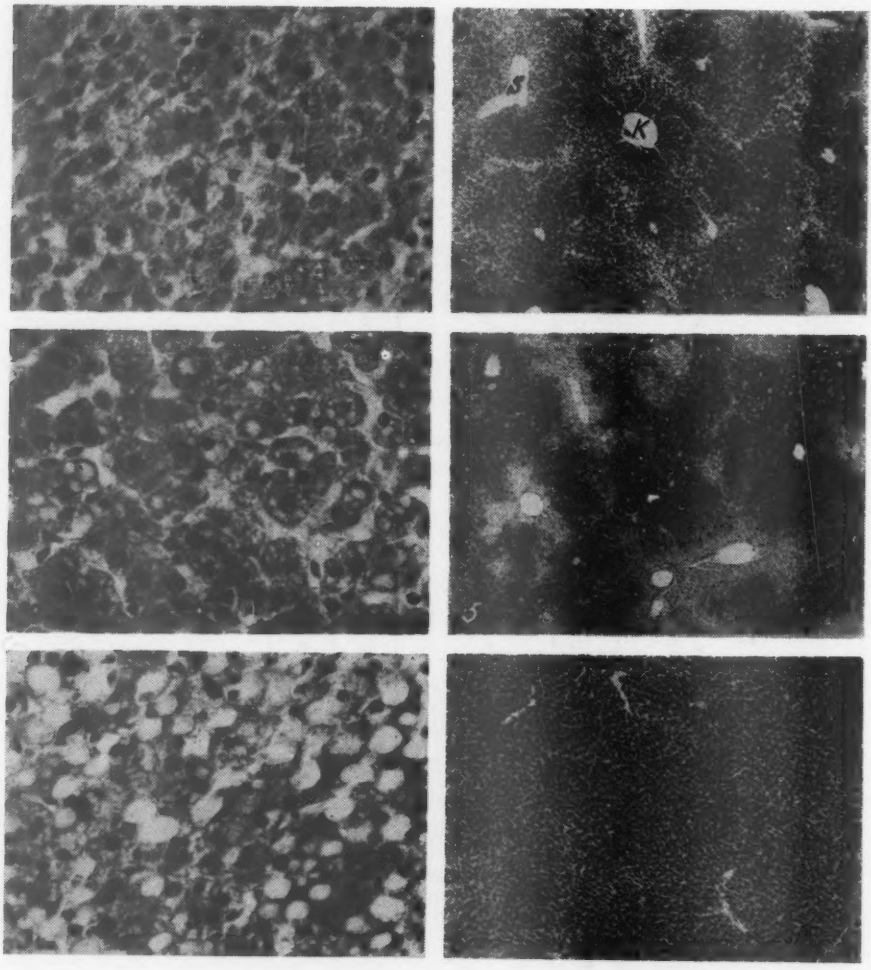

FIG. 2.—*1: Section of the liver of a 3 days old rat, born at Mina Aguilar (3990 m); early stage of fatty infiltration (haematoxylin-eosin, × 450). 2: Section of the liver of a 7 days old rat, born at Mina Aguilar (3990 m); mild fatty infiltration. Hepatic cells with spongy protoplasm (haematoxylin-eosin, × 450). 3: Section of a 17 days old rat. born at Mina Aguilar (3990 m); severe fatty degeneration of the hepatic parenchyma (haematoxylin-eosin, × 450). 4: Section of the liver of a 17 days old rat, born at Mina Aguilar (3990 m); topographical view. Degeneration of the medial zone of the lobule in badge form, the central and peripheral zones are undamaged (haematoxylin-eosin, × 50). K: Kiernan space; S: supralobular vein. 5: The same animal as in 4 (Scarlet red, × 50). 6: Section of the liver of a rat 17 days old, born at Mina Aguilar (3990 m) and maintained since birth at sea level pressure (hyperpressure chamber). Normal structure of the liver (haematoxylin-eosin, × 50).*

Mortality was high during the first three days (35 per cent including the deadborn) ; it decreased afterwards to increase again between the 10th and 17th day of life. In this last period, 38 per cent of the total died.

The average number of young per litter was 7.0; the average body weight at birth, 5.2 g. The newborn presented severe cyanosis, more noticeable on account of the lack of hair, they were cold and not as lively as those born at sea level. Since young of litters born during the night were not counted and weighed until the following morning we do not know how many young may have been eaten by the mothers during the night. However, the normal average of the litter size found at high altitude would indicate that the number of newborn consumed by the mothers was small.

The young were completely covered with hair between the 11th and 13th day ; at sea level it happens between the 8th and 10th day of life. Hair was always thin with areas of alopecia. In most of cases eyes were opened at the 20th day of life; at sea level it occurs between the 14th and the 16th day ([12, 13]). Growth delay, not very noticeable in the first week, became evident when the animals were over 10-12 days old (fig. 1). The lack of proportion between head and body was striking and due to the smallness of the latter. They died suddenly while apparently in good general condition. All intents of artificial feeding after the 8th day of life ended in death of the young. The same results were obtained when solutions of methionine and cisteine were administered by mouth. The injection of vitamin B_{12} proved also ineffective.

The skin was yellow, colour being more noticeable in the hairless regions particularly in the ears. Skin around the sexual organs was stained yellow with the urine pigments, a fact which does not occur at sea level. Autopsy, which was practised in almost all cases, showed a big liver, with blunt borders, friable and yellowish red.

Histological sections of the liver in rats of different ages showed progressive lesions of fatty degeneration. In the three days old rats (fig. 2,*1*), zones with cells of indistinct contours and vacuolated protoplasm are to be found especially in the mid zone of the hepatic lobule; those zones are more or less equidistant between Kiernan's space and the central vein. In the 7 days old rats (fig. 2,*2*), the mentioned zone shows poligonal and frequently spherical cells, with a wreath of protoplasmatic vacuoles, which give them a spongy appearance. These vacuoles contain lipids or neutral fats, as demonstrated by Sudan III and scarlet red stains. Fat vacuoles increase their size rapidly and concur in a sole vacuole that displaces the picnotic nucleus towards one pole of the cell (fig. 2,*3*). The severe fatty degeneration is responsible for the aspect of a badge presented by the hepatic lobule (fig. 2,*4*), particularly remarkable in the sections stained for fat (fig. 2,*5*). This degree of fatty degeneration is generally reached around the 15th to 17th day of life.

The histological study of the liver of seven embryos autopsied a few days before delivery did not show any abnormality.

In order to see whether by suppressing hypoxia death of the new-

borns could be avoided, we placed in the hyperpressure chamber (its details are given in methods) a mother with ten newborn rats. Cyanosis quickly dissappeared in the latter, being replaced by the normal pink colour. They were not cold and got more lively. Growth was similar to that observed at sea level (12, 13) and greater than in the animals reared at the barometric pressure of Mina Aguilar (fig 1). When young were 30 days old they were taken out of the chamber and separated from their mother; 6 months later they were still alive and in good health.

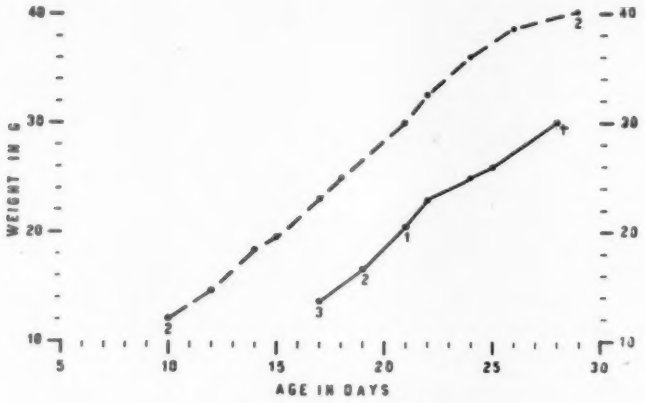

FIG. 3. — *Growth curves of the young rats of two litters born at Mina Aguilar, 3 990 m above sea level.*
— — — kept at barometric pressure, annual average 476 mm Hg.
------ kept under sea level pressure (hyperpressure chamber) since birth to 10th day of life, then nursed at barometric pressure (average 476 mm Hg) by the mother of the other litter, at the same time that her 3 youngs, who were 7 days older.
Numbers on the lines indicate number of animals.

For the histological study, young rats reared in the hyperpressure chamber were killed at regular intervals. In the liver sections small zones of fatty degeneration were found occasionally during the first week of life, accompanied in some cases by signs of parenchyma regeneration. Liver was normal in the older animals.

We consider of interest to describe in detail the following experiment: a mother and her two newborn offsprings were placed in the hyperpressure chamber; the mother died accidentally after 10 days and the young were withdrawn from the chamber and given in adoption to another rat who had three 17 days old young. Of these three young, one died at 19 days, the other at 21 and the last one at 23 days of age. The fact that the two rats that had been in the hyperpressure chamber grew normally being nursed simultaneously with the other three, who evidently grew less

(fig. 3), and died before the 30 days of life with the typical hepatic lesions permits us to discard a faulty lactation as the cause of the growth delay and death of the suckling young at high altitude, as stated by Moore and Price [13]. Our experiment also shows that suppressing hypoxia during the first 10 days of life delays but does not prevent the liver damage, since the two rats that had been in the chamber died at 38 and 45 days of age, respectively.

Discussion

Our findings indicate a fatty degeneration of the liver in young rats born and living at 3 990 m of altitude. Liver damage appeared in most cases during the first 30 days of life and progressed with age killing the animals between 10 to 17 days. Only 12.5 per cent of the young surpassed 17 days of age and 1.6 per cent, 30 days. The hepatic lesion was very mild or did not exist in the newborn.

The litter size and the average body weight at birth were similar to those found at sea level in the same strain of rats. These facts support the existence of a normal intrauterine development of the embryos at Mina Aguilar and discards the possibility of having a higher proportion of fetal resorption than at sea level. Moore and Price [11] arrived to similar conclusions at Mount Evans (4 278 m).

The average litter size of the rats born at Mount Evans was according to Moore and Price [11], smaller than that found by us at Mina Aguilar (3 990 m): 6.0 instead of 7.0; the average body weight being on the contrary a little higher, 5.47 g instead of 5.2 g.

We think that the high rate of mortality at birth and during the first days of life of the newborn rats is due to a greater hypoxia at the time of delivery and perhaps to defficiencies in the adaptation mechanisms of the newborns.

The development changes observed in our animals: delay of growth, delay in the appearance of hair and in the opening of eyes and also the sudden death occurred while the young were in a fairly good general condition, can be explained by the hepatic lesion.

The experiment of nursing the two rats that had remained the first 10 days of life in the hyperpressure chamber, by the same mother than the other three reared at the normal barometric pressure of Mina Aguilar, permited us to discard a faulty lactation as a cause of death or growth delay in the young born at this place. In fact, the two adopted young rats would not have grown normally if the poor development of the other three young, 7 days older, were due to an impaired lactation. On the other hand, although the two adopted young had enough food for a normal growth it did not prevent the liver damage since they died at 38 and 45 days of age, respectively.

The fact that the animals submitted during the first 30 days of life to an oxygen pressure similar to that at sea level do not present hepatic

lesion and survive normally, confirms that hypoxia is the cause of the lesion.

We ignore which is the shortest time in which the newborn rats should not suffer from hypoxia in order to have a normal survival, but we found that suppressing the hypoxia during the 10 first days of life delayed death a few days only.

It is striking to observe that rats exposed to hypoxia after the 30 or 40 days of age endure it relativelly well, the opposite to what happens with the younger ones. This fact has been observed by many authors and is confirmed by the behaviour of the 10 rats born at Mina Aguilar, reared during the first 30 days of life at sea level pressure (hyperpressure chamber), who thereafter continued to live in good health at the barometric pressure of Mina Aguilar (anual average, 476 mm Hg).

The adult rats brougth to Mina Aguilar did not present hepatic lesions nor a higher death rate than at sea level during the observation period of this work. Fatty degeneration of the liver, in rats, mice, guinea pigs and rabbits have been described ([14, 15, 16, 17, 18, etc.]), but they are concerned with much higher simulated altitudes than that of Mina Aguilar (3 990 m).

Regarding the mechanism of production of the hepatic lesion it cannot be ascribed to a lack of lipotropic factors since their administration was ineffective. Nevertheless we cannot be conclusive about it since we administered the lipotropic factors after the 8th day of life when the hepatic lesion was already well established and also on account of the small number of our observations.

Altland and Highman ([19]) did not observe a profitable effect of methionine on the pathologic alterations produced by hypoxia in rats exposed 4 hours daily during 10 weeks, to a barometric pressure of 282 mm Hg.

SUMMARY AND CONCLUSIONS

Growth and development of albino rats born in Mina Aguilar, 3 990 m above sea level has been studied. Hypoxia present at this altitude produced:

a) a fatty degeneration of the liver during the first 3 days of life that prevented survival of the rats born there.

b) delay of growth and development as a result of the liver damage.

By suppressing the hypoxia during the first 30 days of life, the hepatic lesion was prevented and the animals survived normally.

REFERENCES

([1]) ALTLAND, P. D.: *Anat. Record*, 1947, *99*, (suppl.). 32.
([2]) ALTLAND, P. D.: *J. exper. Zool.*, 1949, *110*, 1.

(3) ALTLAND, P. D.: *Physiol. Zool.*, 1949, *22*, 235.
(4) HIGHMAN, B., ALTLAND, P. D.: *Arch. Pathol.*, 1949, *48*, 503.
(5) ALTLAND, P. D., ALLEN, E.: *Anat. Record*, 1950, *108*, 609.
(6) ALTLAND, P. D.: HIGHMAN, B.: *Amer. J. Physiol.*, 1951, *167*, 261.
(7) MALMÉJAC, J., ROSSI, M., PLANE, P.: *C. R. Soc. Biol., Paris*, 1949, *144*, 271.
(8) NELSON, D., BURRILL, M. W.: *Fed. Proc.*, 1944, *3*, 34.
(9) SUNDSTROEM, E. S., MICHAELIS, G.: *Mem. Univ. California*, 1942, *12*, 1.
(10) ACCAME, O. F.: *Anales Fac. Med. Lima*, 1945, *28*, 65.
(11) MOORE, C. R., PRICE, D. J.: *J. exper. Zool.*, 1948, *108*, 171.
(12) CHIODI, H.: *El timo en relación con el crecimiento y la función sexual.* Tesis Fac. Med. Buenos Aires, Edit. El Ateneo, 1938, pág. 130.
(13) FOGLIA, V. G. GÜNTHER, B., PINTO, R.: *Rev. Soc. Argent. Biol.*: 1943, *19*, 384.
(14) MARTIN, G. H., BUNTING, C. H., LOEVENHART, A. S.: *J. Pharmacol.*, 1916, *8*, 112.
(15) ROSIN, A.: *Beitr. pathol. Anat. u. allg. Pathol.*, 1927, *76*, 153.
(16) LOEWY, A., LEIBOWITZ, J.: *Bioch, Ztschr.*, 1928, *192*, 67.
(17) LUFT, U.: *Beitr. pathol. Anat. u. allg. Pathol.*, 1937-38, *98*, 323.
(18) CHEVILLARD, L., HAMON, F., MAYER, A.: *Ann. Physiol.*, 1937, *13*, 634.
(19) ALTLAND, P. D., HIGHMAN, B.: *Anat. Record*, 1950, *108*, 608.

METHOD OF ASSAY OF BRADYKININ UPON THE GUINEA PIG GUT

M. ROCHA E SILVA

(Department of Biochemistry and Pharmacodynamics, Instituto Biológico, São Paulo, Brazil)

IN PREVIOUS papers from this laboratory ([1, 2]) we have described experiments on the release and partial purification of a hypotensive and smooth muscle stimulating principle —bradykinin— by incubation of normal plasma globulins with the venom of *Bothrops jararaca* or with crystalline trypsin. In the course of the experiments of purification it was found advisable to develop a method for the quantitative assay of this principle. The smooth muscle of the guinea pig gut is far the most convenient biological structure for bradykinin assay. As described previously, bradykinin produces a slow contraction of the gut, contrasting with the quick, almost instantaneous effect produced by histamine or acetylcholine. When time-concentration curves are drawn bradykinin can be shown to produce a given effect (same height of contraction) about 6 to 7 times as slowly as histamine. This constituted the reason to name the substance *bradykinin,* indicating a substance that produces a slow movement of the gut (from *bradys* + *kinesia*).

To measure the effect of bradykinin upon the gut, the height of the contraction registered upon a smoked drum with a linear frontal lever, is taken in millimeters. In a certain phase of the work, it was thought that for measuring the effect, one should have to measure both the height and the slope of the contraction curve, or better, the area between the curve and the time axis, after a fixed interval of time of contraction. From the analysis presented below, this was found unnecessary, since the slope is correlated to the height, and the latter is linearly related to the logarithm of the dose and therefore constitutes an appropriate measure of the effect.

At the beginning, the method used in our laboratory for estimating bradykinin potency, was the usual method for assaying histamine upon

Received for publication October 20, 1952.

the gut (3) or pitocin upon the uterus (4). The standard solution of known potency was repeatedly added to the bath bracketing increasing doses of the unknown until a perfect matching of standard and unknown was obtained. As stressed by Schild (5) this method is unsatisfactory for two reasons: a) only one of the responses that matched with the standard is employed to avaliate potency; b) the procedure does not lead to any statistical treatment of the data. Especially the error of the assay is very difficult to estimate in the classical method of matching standard and unknown.

The experiments presented in continuation were done with the aim of devising a method based on the principles of modern biological standardization for assaying bradykinin leading to an appropriate calculation of the confidence limits of the ratio of potency between standard and unknown. A test for the linearity of the dose-response curve is also included.

Material and Methods

Bradykinin was prepared according to the method previously described (1). In most of the experiments presented in this paper we have made use of a crude bradykinin, labelled *Pool I*, a homogenate of many batches of crude bradykinin. Its potency is almost twice the potency of *Brady I*, as used previously as standard (1). The activity of *Pool I* was taken as *one unit of bradykinin per mg weight.*

The assay was performed in quite standardized conditions by taking a piece of ileum from about 2 to 3 cm and suspending it in a Tyrode bath of 7 cm^3 capacity after the introduction of the gut. The writing lever used was a frontal one, with a long writing point, as described by Schild (6). Unless excursions of the lever go beyond an angle of 30°, inscription does not depart from linearity for more than 3 %.

Before starting any assay, the muscle was made to contract with several additions of bradykinin, until it gave fairly comparable results following the addition of the same dose of the active material. The interval from washing out the muscle with new Tyrode solution and the addition of the following dose of bradykinin was 3 minutes, since it was found that repeated additions in shorter intervals, produced a decrease in response and finally a temporary blocking of the muscle toward any further addition of bradykinin.

A preliminary study of the dose-response curve indicated that a straight line relationship exists between the log dose and the response. Therefore, the results can be obtained in terms of a log ration of potency, as indicated below.

Te assay for estimating the log ratio of potency was designed in such a way to eliminate variation due to changes of sensitivity along the assay. For this purpose, two doses of the standard and two doses of the unknown were added at random, and the four doses to be added are chosen in previous testing in order to have a set of doses giving small,

medium and sub-maximal responses of the gut. One of the pitfalls of the method is to employ doses that might fall inside the range producing maximal contraction of the gut. The ratio of activity between the two doses of the standard were the same as between the two doses of unknown. Usually we have taken doses of standard and the doses of unknown in a common ratio 1:2. The main objective of the assay is to find the ratio of potency between unknown and standard. This ratio should not exceed

TABLE 1

Curve log-dose-effect with three doses of bradykinin

Groups:	S_1	S_2	S_3	Doses in tenths of units
	2.00	3.00	4.50	Polynomial coefficients
	−1	0	+1	
				Totals (T_g)
I	28	43	55	126
II	24	45	68	137
III	15	33	46	94
IV	15	35	50	100
V	18	32	45	95
Totals T_d	100	188	264	T = 552
				$\overline{Y} = 36.8$

Total sum of squares: $SY^2 - \frac{Y^2}{N} = 3322.4.$

Sum of squares *between groups* = 521.7.

the ratio between the two doses of the standard and of unknown, but it is convenient that it does not differ considerably from 1. In the assays performed in this paper, the ratio of potency was taken as 1.25. The four sets of doses in one of the dummy experiments performed to test the method were as follows, in tenths of "units" (mg of *Pool I*):

$U_2 = 2.5$ $S_2 = 2.00$
$U_1 = 1.25$ $S_1 = 1.00$

Since we can assume that for the range of doses employed the responses of the gut are linearly related to the logarithm of the dose, as shown in the first experiment presented below, and the two doses of unknown and standard equally logarithmically spaced, a great simplifica-

tion can be introduced by taking polynomial coefficients instead of the doses and adjusting the figures for doses of unknown to those of doses of standard:

$$U_2 = +1 \qquad S_2 = +1$$
$$U_1 = -1 \qquad S_1 = -1$$

Assuming a common potency for both solutions, the ratio of potency to be found as a result of the assay will appear as a correction term for the assumed potency of the "unknown". With the use of polynomial coefficients, the log ratio of potency (M) will be found in terms of a logarithm the bases of which will be equal to the ratio of doses. In our case, this ratio was 2, therefore the convertion to $\log_{10}$ will be made by multiplying the results by $\log_{10} 2 = 0.30103$. This is the meaning of I, in the formulas (I) and (II).

TABLE 2

Coefficients for doses:				Divisor	Sum of products	Variances $\frac{(SkT_d)^2}{n_d Sk^2}$
Linear	−1	0	+1	10	164	2689.6
Quadratic	+1	−2	+1	30	−12	4.8
Total T_d	100	188	264	—	—	—

The symbolism adopted in this paper was taken from Emmens (7). The log ratio of potency (M) can be estimated either graphically (fig. 1) or by applying the formula:

$$M = \frac{I\,D}{B} \qquad (I)$$

where $I = 0.30103$. The values indicated as D and B will appear as the square roots of the variances corresponding to "variation between samples" (D^2) and "variation due to linear regression" (B^2) in the analysis of variance, as shown in Tables VI and VIII.

The approximate confidence limits for M can be found by adding or subtracting t times (the value of *t* is taken from a "Student's" table for the corresponding degrees of freedom associated with the error of the assay and the desired level of probability) the standard error of M (s_M) to or from M, according to the formula:

$$\text{Confidence limits of } M = \text{Antilog } (M \pm t\, s_M)$$

The standard error of M will be estimated by the approximate formula (II):

$$s_M = \frac{s\, I \sqrt{D^2 + B^2}}{B^2} \qquad \text{(II)}$$

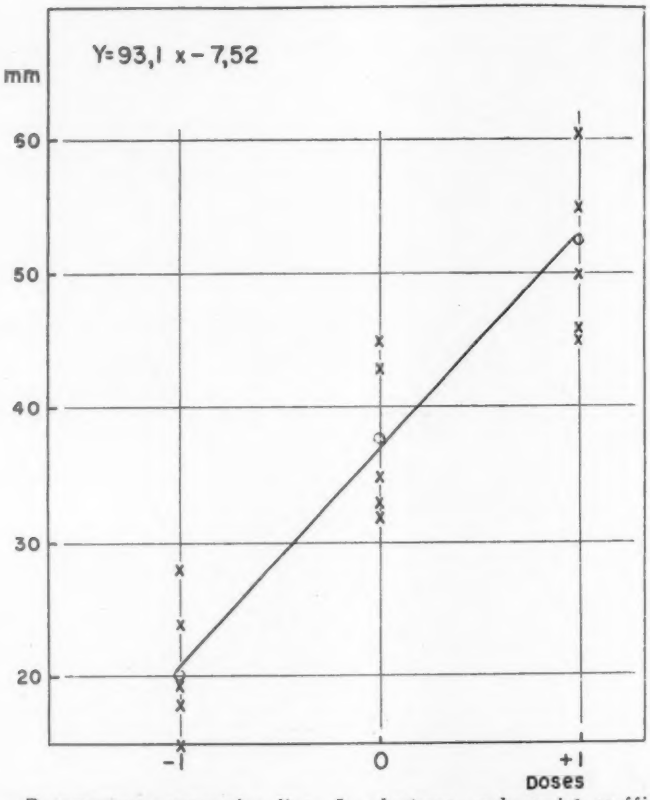

FIG. 1. — *Dose-response regression line. In abscissae: polynomial coefficients for doses. Ordinate: height of contraction in millimeters. In the equation above, Y, indicates the responses and x the* log_{10} *doses. The abscissae con be transformed into the base 10 logarithmic scale, making* $-1 = 301 = log_{10}2$; $0 = 0.477 = log_{10}3$ *and* $+1 = 0.653 = log_{10}4.5$.

where *s*, represents the square root of the *error* as obtained in the analysis of variance, I = 0.30103 and D and B, retain their previous meanings.

RESULTS

Deviation from linearity. A preliminary assay was performed to test the linearity of the curve relating intensity of response and dose as

expressed in a logarithmic scale. Three doses of the substance were added in the ratio 1:1.5, in a randomized way, in five groups of three. The responses in mm are presented in Table 1.

The *total* sum of squares was found to be $Sy^2 = 3\ 322.4$ and the sum of squares *between groups* was found to be 521.7. Using polynomial coefficients (Table 2), we can easily calculate the sum of squares corresponding to "linear" regression and "deviation from regression" (Quadratic). The sum of squares *between* doses is the total of the two parcels obtained in Table 2, and equal to 2 694.4.

TABLE 3

Analysis of variance of data of Table 1

Source of variation	Degrees of freedom	Sum of squares	Variances	F	P
A. Regression line	1	2 689.6	2 689.6	224.8	< 0.001
B. Deviation from regression	1	4.8	4.8	0.37	> 0.05
C. Between Groups	4	521.7	130.4	9.7	< 0.05
Error [T—(A+B+C)]	8	106.3	13.3		
Totals	14	3 322.4			

After estimation of the slope $b = 164/10 \times 0.1761 = 93.1$, the regression line is given by the equation:

$$Y = 36.8 + 93.1\ (X - 0.477)$$

As shown in fig. 1, the response to bradykinin follows very closely a straight line. The significance of the regression and the non-significance of the deviation from linearity are shown in Table 3 in which the analysis of variance of data presented in Table 1 is presented.

The ratio of variances ($F = 224.8$) for the regression line is far above the 99.9 % level of probabilities, the corresponding ratio ($F = 9.7$) for variation "between groups" is significant, this indicating a substantial variation of sensitivity of the gut during the assay. "Deviation from regression" is below the experimental error and therefore non-significant. The *standard error* of the assay $s = \sqrt{13.3} = 3.65$ represents about 10 % of the mean $\bar{Y} = 36.8$ mm for all determinations.

The four point assay. As indicated before, the log ratio of potency (M) between two solutions ("Unknown" and Standard) can be calculated

TABLE 4

Assay of potency with 2 doses of standard and 2 doses of unknown

Doses	Units	Responses in mm Groups					Totals
		I	II	III	IV	V	T_d
U_2	0.50	52	50	49	45	44	240
U_1	0.25	31	31	25	27	23	137
S_2	0.40	45	49	43	36	44	217
S_1	0.20	22	26	23	20	19	110
Totals T_g		150	156	140	128	130	704 = T
							$\overline{Y} = 35.2$

Total sum of squares $Sy^2 = 2\,547.2$.

Sum of squares *between groups* = 149.2.

Sum of squares *between doses* = 2 330.8.

TABLE 5

Analysis of variance of data of Table IV

Source of variation:	Degrees of freedom	Sum of squares	Variances	F	P
A. Between groups	4	149.17	37.29	6.7	< 0.05
B. Between doses	3	2 330.80	776.6	139.0	< 0.001
C. Error [T—(A+B)]	12	67.23	5.6		
Totals	19	2 547.20			

when two quantities D^2 and B^2 are known. These quantities are given by the numbers indicating the mean sum of squares (variances) for "Standard" x "Unknown" and "linear regression". In the experiment given in Table 4, two doses of the "unknown" (0.50 and 0.25) and two doses of the standard (0.40 and 0.20 units) were assayed in groups of 4, each containing the four doses in a randomized order. The analysis of variance can be set as indicated in Table 5.

The three degrees of freedom available for the sum of squares "between doses" can be used up to split it into three parcels, as indicated in Table 6. These three parcels corresponding each one to one degree of

TABLE 6

Partition of the sum of squares corresponding to variation "between doses' of table 5

Source of variation:	Factorial coefficients: S_1	S_2	U_1	U_2	Divisor nSk^2	Sum of products Sk^2T_d	Variances
A. Between samples	−1	−1	+1	+1	20	+ 50	125 D^2
B. Linear regression	−1	+1	−1	+1	20	+210	2 205 B^2
C. Dev. from parallel.	+1	−1	−1	+1	20	− 4	0.8
Totals (T_d)	110	217	173	240			
					Error = 5.6	12 d.f.	

freedom, are respectively: a) mean sum of squares between samples ("unknown" and standard) = D^2; b) mean sum of squares corresponding to "linear regression" = B^2; c) mean sum of squares corresponding to "deviation from parallelism". They total 2 330.8, as indicated in Table 5.

The average slope of the combined regression line can be calculated by applying the formula:

$$b = \frac{2SkT_d}{InSk^2}$$

where SkT_d = the sum of products corresponding to linear regression

(= 210) and nSk^2 = the divisor (= 20) and I = 0.30103.

$$b = \frac{2 \times 210}{0.30103 \times 20} = 70{,}0$$

If the two doses of the standard and "unknown" are conventionnally given as 2 and 4 units, their respective logarithms in base 10, will be

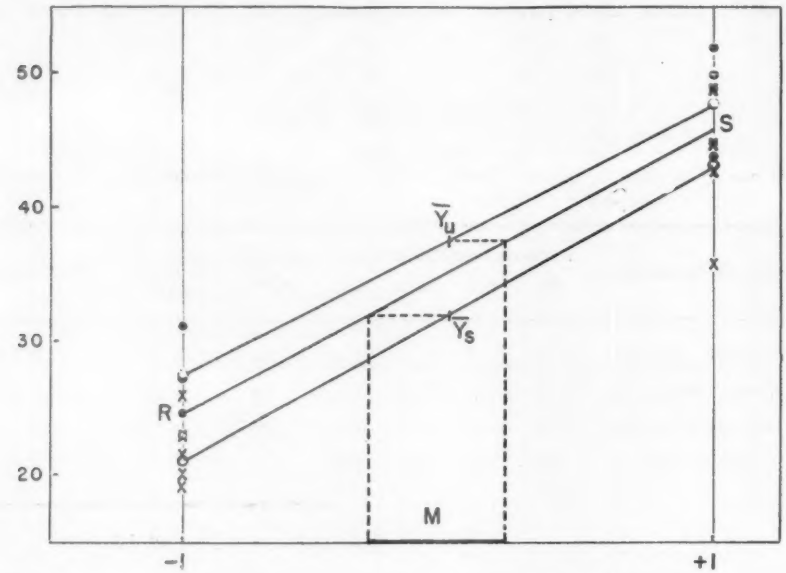

FIG. 2. — *The mean regression line (RS) lies between the two regression lines for unknown (U) and standard (S). Abscissae: factorials for doses. (.) indicate individual responses to both doses of unknown, (x) indicate the corresponding responses to the standard and (o) indicate the mean responses to the larger and smaller doses of unknown and standard. M, the horizontal distances of the two regression lines indicates the log-ratio of potency.*

0.301 and 0.602, with an average $\bar{X} = 0.452$ and the combined regression line (R, S) will be:

$$Y = 35.2 + 70.0\ (X - 0.452)$$

or

$$Y = 3.56 + 70.0\ X$$

In fig. 2, the ratio of potency (M) can be graphically estimated by measuring the distance M. The geometrical construction is obvious, since

the slope of the average regression line is squal to $\bar{Y}_u - \bar{Y}_s / M$ and, therefore,

$$M = \frac{\bar{Y}_u - \bar{Y}_s}{b}$$

The estimation of the log ratio of potency can, however, be done, more precisely by applying the equation:

$$M = \frac{I\,D}{B} = \frac{0.301\sqrt{125}}{\sqrt{2\ 205}} = 0.07173$$

and the standard error of M_{10} can be calculated through the equation:

$$s_M = \frac{s\,I\sqrt{D^2 + B^2}}{B^2} = \frac{\sqrt{5.6 \times 0.301} \times \sqrt{125 + 2\ 205}}{2\ 205}$$

The approximate limits of error of M, will be given as antilog $(M \pm 3.05\, s_M)$ taking $t = 3.055$ from a table for distribution of "Student's" t, for a 99 % level of probability. The ratio of potency U/S will be equal to 1.18 with confidence limits 1.32 — 1.06 for a 99 % level of probability. It is clear that the real ratio of potency (1.25) falls confortably between the confidence limits calculated from the experimental data.

The geometrical construction given in Fig. 2 gives more rapidly this ratio of potency, but the error of the assay can only be obtained through the usual routine analysis of variance as indicated in Table 5. We have therefore to calculate separately the *total* sum of squares and the sum of squares *between groups*. The sum of squares *between* doses is more easily calculated by applying factorial coefficients as indicated in Table 6. The values for D and B, are given immediately by applying the scheme of calculation indicated in Table 6.

The advantage of the design. If the experiment was not so designed as to eliminate heterogenity *between* groups (mostly due to drop of sensitivity), the sum of squares *between* groups, with 4 degrees of freedom, would appear in the parcel corresponding to error, with 16 degrees of freedom, this would reduce substantially the precision of the experiment presented in Table 5. In fact, the precision of an assay can be defined as the reciprocal of the variance of the error if the slope is constant. Without the proper design, the variance of the error of the experiment of Table 5, would be

$$(149.17 + 67.23) / 16 = 13.52$$

instead of 5.6, this indicating a drop of about 2.4 in the precision of the assay.

Table 7 shows details of several assays of potency performed with known solutions of standard and "unknown". The 6 ratios (found) averaged 1.23 that constitute an excellent measure of the real ratio (1.25). The ratio s/b, introduced by Gaddum ([8]), between the standard error (s)

TABLE 7

Latin square

C 17	D 17	A 46	B 39
A 46	B 41	C 30	D 24
D 18	C 32	B 46	A 48
B 41	A 47	D 25	C 32

Doses in "units"

A = 0.25 (U_2)
B = 0.20 (S_2)
C = 0.125 (U_1)
D = 0.10 (S_1)

TABLE 8

Analysis of variance of data of Table 7 (Latin square)

Source of variation:	Degrees of freedom	Sums of squares	Variances	F	P
Between rows (groups)	3	112.50	36.8	6.6	< 0.05
Between columns	3	90.19	30.06	5.5	$\cong 0.05$
Between doses:					
Standard × Unknown	1	138.06	138.06 D^2	24.7	< 0.01
Linear regression	1	1 580.06	1 580.06 B^2	1 580.06	< 0.001
Dev. from parallel.	1	3.06	3.06	0.56	
Totals	15	1 957.44			

of the method and the slope of the average regression line (b), given in the last column of table 9 is an absolute measure of variability and tends to stabilize when the assay is properly performed. It is clear that but for the values of experiments III and V, this ratio had a rather narrow range between 0.044 and 0.033. The two discrepant values for experiments III and IV might be due a) to the small number of groups used, 2 in exp. III and b) to performance of the assay in the range of maximal contractions of the gut, as occurred in some of the groups presented in exp. V. Experiments V and VI of Table 7, were designed as a *Latin square*. In those cases the *order* of addition inside of the groups could also be checked and the sum of squares corresponding to variation of sensitivity "inside of the group" calculated.

Discussion

The purpose of any biological assay is to estimate the ratio of potency of two solutions, one of "unknown" composition to be compared with the standard. To such an estimate is attached an "error" that must be deduced from the inherent variation of the biological material employed in the assay. This obviously depends upon the many conditions under which the assay has been performed. The advantage of adopting a proper *design* for the performance of the assay is to reduce this error to a minimum compatible with the nature of the material employed and the conditions prevalent in the laboratory where the assay is being routinely performed. The usefulness of the so-called 4-point design for standardization of biological materials has been repeatedly emphasized by several workers. Bliss and Marks (9) employed such a design to estimate the potency of insulin preparations and Schild (5) presented a full account of the method to estimate histamine. More recently, Holton (10) used the same design as described by Schild for assaying pituitary ocytocic principle upon the rat uterus.

The advantage of the 4-point assay, besides its simplicity, is that even when parallelism between standard and unknown is not strict, the method can give a reliable indication of the ratio of potency. We quote Finney (11): "when the "parallelism" component in the 4-point analysis of variance is not significant there is no reason for alarm, but when it is significant the cause may be invalidity of statistical analysis and it is not necessarily a sign of complete invalidity of the assay". In the case of the assay described above, as in histamine assays described by Schild, the analyst is comfortably in the safe side, since the responses of the gut do not significantly depart from linearity in a wide range of log-dose variation, and deviation from parallelism between standard and unknown is usually not significant.

References

(1) Rocha e Silva, M., Beraldo, W. T. and Rosenfeld, G.: *Amer. J. Physiol.*, 1949, *159*, 261.

(2) Rocha e Silva, M.: *Bradicinina. Preparação, Ensaio biologico e Identificação.* Tese Fac. Nac. Medicina, 1951.

(3) GUGGENHEIM, M., LOEFFLER, W.: *Biochem. Z.*, 1916, *72*, 303.
(4) BURN, J. H.: *Biological Standardization.* Oxford Univ. Press., 1937.
(5) SCHILD, H. O.: *J. Physiol.*, 1942, *101*, 115.
(6) SCHILD, H. O.: *J. Physiol.*, 1944, *103*, 11 P.
(7) EMMENS, C. W.: *Principles of Biological Assay.* Chapman and Hall, London, 1948.
(8) GADDUM, J. H.: *Biochem. J.*, 1941, *25*, 1113.
(9) BLISS, C. I. AND MARKS, H. P.: *Quart. J. Pharmacy and Pharm.*, 1948, *12*, 82.
(10) HOLTON, P.: *Brit. J. Pharmacol.*, 1948, *3*, 358.
(11) FINNEY, D. J.: *Statistical Analysis.* In: BURN, J. H.: *Biological Standardization.* Oxford Univ. Press, 1950.

BIOASSAY OF HYPERTENSIN IN PITHED RATS AND CATS BASED ON A FOUR-POINT DESIGN *

J. R. VALLE, ELINE S. PRADO (**) AND J. L. PRADO

(Laboratórios de Farmacologia e Bioquímica da Escola Paulista de Medicina e dos Centros de Saúde da Capital, São Paulo, Brasil)

HYPERTENSIN has been assayed currently in intact dogs or in pithed and intact cats by the usual method of comparing the blood-pressure raising effect of a known solution with different amounts of the unknown solution so as to obtain close responses. This procedure of assaying biologically active substances is far from satisfactory. The great variability in blood-pressure responses observed in the same pithed animal injected repeatedly with equal doses of hypertensin limits the precision of the assay and in such experimental conditions even the calculation of the error of the method may be misleading. Schild (1942) in a well known paper about the quantitation of histamine has given good reasons for employing the type of assay we are following in the present paper. His design has been already adopted by different investigators such as Valle, Henriques and Henriques (1947) for androsterone, Holton (1948) for the ocytocic potency of pituitary extracts and Rocha e Silva (1950, 1951), for bradykynin.

Although other biological structures have been employed to recognize or even to estimate hypertensin (Page, 1942; Fasciolo and Taquini, 1947) the suggestion advanced by Shipley and Tilden (1947) that the pithed rat might be suitable for assaying hypertensin seemed very attractive. This paper will describe our results by applying the principles established

---

* This work was subsidized by a grant from the Conselho Nacional de Pesquisas, Brasil. Read before the Seminários de Estadística Aplicada, São Paulo, June 26, 1952 and the Sociedade de Biologia de São Paulo, July 17, 1952.

** With a fellowship from the Conselho Nacional de Pesquisas, Brasil.

Received for publication October 20, 1952.

by Schild and using as test preparations the arterial blood-pressure of pithed cats and rats.

MATERIAL AND METHODS

a) *Pithed cat.* Cats of both sexes weighing from 1.3 to 4.0 kg were used. After pentobarbital anesthesia (30 mg/kg intraperitoneally) and subcutaneous injection of atropine (5 mg per animal) the method of pithing described by Shipley, Helmer and Kohlstaedt (1947) was exactly followed. Carotid blood pressure was recorded with a mercury manometer and injections were made through a plastic tube (*) inserted in one of the femoral veins.

b) *Pithed rat.* The procedure of Shipley and Tilden (1947) was used. Male adult rats 200-300 g body weight were employed. The animal is pithed by introducing through the orbit into the deeply anesthetized animal (pentobarbital intraperitoneally 50 mg/kg) a blunt rod sufficiently long to enter the whole length of the vertebral canal. Previous to the pithing the rat was atropinized (5 mg/kg) and had both vagi cut. Artificial respiration was obtained with a respirator system consisting of a 10 ml hypodermic syringe connected with a one way metallic valve and moved by a record playing motor. Both speed and amplitude of the plunger excursion could be easily regulated.

Both preparations, the cat and the rat, were kept warm (rectal temperature at about 35°C) throughout the assay the animals being used only 30 to 40 minutes after pithing.

c) *Hypertensin solutions.* Different small samples of hypertensin, received from various investigators or prepared in this laboratory, which varied extremely in potency, were asseyed. The brown crude preparation from Dr. P. Edman, will be called *Edman I;* the limpid solution from Dr. O. M. Helmer, assaying according to his estimate 20 u./ml, will be known as *Helmer's* and the first batch of hypertensin prepared by one of us (E. S. P.) will be referred to as EPI (**). From the sample to be employed hypertensin solutions of different concentrations were prepared so as to permit injections of *different* doses dissolved in the *same* volumes (0.2 — 0.3 ml).

d) *Dose-response-relationship.* As a preliminary step we had to study the relationship between the injected dose of hypertensin in ml, mg or μg and the obtained increase of the blood pressure in mm of mercury. Different doses of hypertensin keeping among them a constant relation, usually 1:2, and covering a sufficiently large range of responses, were

---

* The polyethylene tubing which has been use in this laboratory was kindly supplied by Dr. George A. Emerson.

** We wish to thank Drs. P. Edman, O. M. Helmer and M. Rocha e Silva, through whom Edman's hypertensin was obtained. To Dr. E. Braun-Menéndez we are especially indebted for having supplie us with a small amount of hypertensin which served to introduce us into this problem.

chosen. Three, four or five doses were thus selected to constitute a group. The order of injections within the group was casualized and they were given in succession with fixed intervals of 5 to 7 minutes, usually enough for the blood pressure to fall to the previous level. Following an interval of 10 or 15 minutes a second group of injections was repeated and the assay continued up to 4 to 6 groups of injections. The blood-pressure raising effects were then measured on the graphic record and the and statistically analyzed as will be shown.

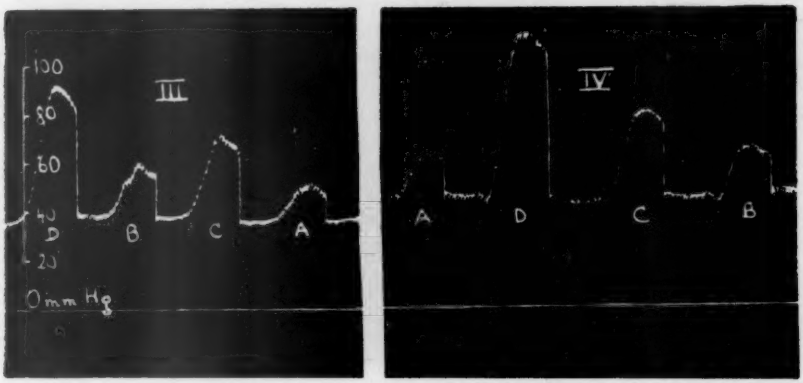

FIG. 1. — *Pithed cat's blood pressure. Response to different doses of Hypertensin. Time between injections 5 minutes, between groups 10 minutes. A = 75 μg; B = 150 μg; C = 300 μg; D - 600 μg of EPI. In this figure are shown two out of a series of six groups.*

e) *Performance of a four-point assay.* By employing a narrow zone of the log-dose-response line, two doses of a "standard" preparation were chosen so that a relationship between them usually of 1:2 was obtained. These two doses are known as S_1 and S_2. Two doses of the "unknown" solution giving a response similar to the two doses of the "standard", and having between them the same relationship as S_1 and S_2, were also selected; they are called U_1 and U_2. S_1, S_2, U_1 and U_2 constitute one group of doses. The order and interval of injections within each group and the interval between groups, were as described above. Four to five groups, usually four, were recorded and the increases in blood pressure measured and statiscally analyzed as will be shown.

There is no need for a lenghty presentation of the procedure and steps of the calculations followed, because they are available even in excellent book form (Emmens, 1948; Burn, 1950).

RESULTS

Log-dose-response-relationship. Figures 1 and 2 show typical records of the effects of repeated injections of different doses of hypertensin in pithed cats and rats. Following a short latent period there is a not too

rapid increase in the blood pressure, wich returns to the previous level in about 3 to 5 minutes, depending on the dose injected. In our records, once the blood pressure had reached its maximum and started the drop,

TABLE 1

Blood pressure increases in mm Hg

Groups	EPI hypertensin in μg			Tg
	150	300	600	
1	9	15	22	46
2	14	20	28	62
3	12	18	34	64
4	23	22	46	91
5	29	34	52	115
TOTAL Td	87	109	182	378

Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean Square	F	P
Linear regression	1	902.5	902.5	63.24	< 0.001
Deviations from regression	1	86.6	86.6	6.06	> 0.2
Between Groups	4	995.0	248.7	17.42	< 0.001
Error	8	114.2	14.27	—	—
TOTAL	14	2 098.3			

the drum was stopped because we were interested only in the maximum height attained. Table 1 shows the analysis of variance of the results obtained in one cat. The deviations from linearity were not significant, which means that within the range studied there is a linear relationship

between the blood-pressure increase and the log dose. In three rats studied we observed a linear relationship even for a more extended range of doses. In the cat, however, if one extends the zone shown in table I, a significant deviation from linearity will be observed. In five cats studied in the same range of doses (fig. 3) the results were equivalent, that is, a linear log-dose-response-relationship was obtained. Probably if one extends sufficiently the range of doses injected in the rat a deviation

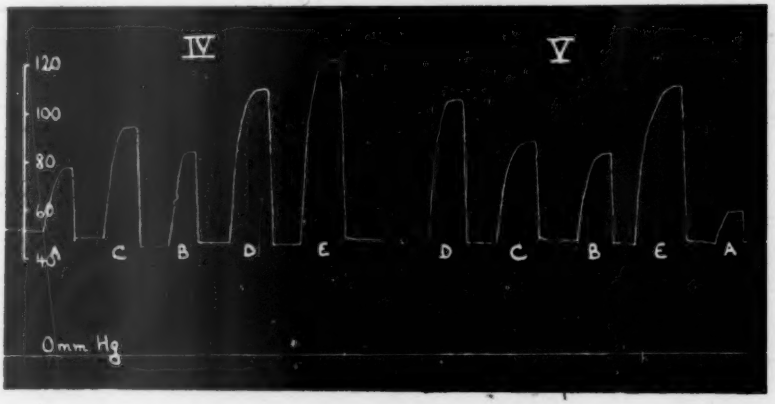

FIG. 2. — *Pithed rat's blood pressure. Responses to different doses of Hypertensin. Time between injections, 6 minutes; between groups 15 minutes.* $A = 0.25$; $B = 0.50$; $C = 1.0$; $D = 2.0$; $E = 4.0$ *mg of Edman's Hypertensin. This figure shows two out of a series of five groups.*

from linearity will also appear, but we needed rather a linear portion of the log-dose-response-curve and, consequently, did not insist on this point.

Four-point-assay. A sufficiently ample zone of linear relationship between dose and response having been selected, we proceeded by performing four point assays into this zone with hypertensin solutions of known strength. Following a few preliminary injections to determine approximately the potency of the "unknown" solution in terms of the "standard", we runned the whole assay. Figures 4 and 5 show typical results in the cat and in the rat. Here also the drum was stopped soon after the blood pressure started its decline. Table 2 shows the analysis of variance of one of such assays. Table 3 gives the summary of the data belonging to a series of hypertensin assays in the rat and in the cat. As may be seen from this table, reasonably good estimates of potency are obtained with the design used. The precision attained with the cat, however, is better than with the rat, which could be easily deduced by comparing the values for λ given in the last column. It will also be observed that a desviation from parallelism was found in only one instance, in the rat, but the estimation of potency obtained in this particular assay was not

highly influenced. It seems, therefore, that the estimation of potency with this design is not too sensitive to slight deviations from parallelism.

TABLE 2

Blood presure increases in mm Hg

Doses of hypertensin in units		GROUPS I	II	III	IV	Td
U_2	1.6	40	59	62	53	214
U_1	0.8	25	26	29	24	104
S_2	2.0	46	62	65	65	238
S_1	1.0	28	45	36	32	141
Tg		139	192	192	174	697

Analysis o variance

Source of variation	Degrees of freedom	Sum of squares	Mean Square	F	P
UXS	1	232.5	232.5	9.1	< 0.05
Linear regression	1	2 678.0	2 678.0	105.4	< 0.001
Deviation from parallelism	1	10.5	10.5	0.4	> 0.2
Between groups .	3	478.0	159.3	6.36	< 0.05
Error	9	229.0	25.4	—	—
TOTAL	15	3 628.0			

The analysis of variance detected a significant variation *between groups* in all the assays; this is a demostration of the necessity of conducting the assay in groups which segregates the source of variation due to alterations of the animal sensitivity during an assay. A latin-square design was tried in two instances with no improvement in the precision.

TABLE 3

Four-point assay of hypertensin solutions from blood pressure increases of pithed rats and cats

Assay	Number of Groups	Test of Significance (F) for:			Actual U/S	Estimated U/S	Error of Estimate %	Limits of Estimate for P = 0.05	S/b = λ
		U x S	Linear Regression	Desviations From Parallelism					
RATS									
Feb. 16	5	**	**	0	1.20	1.32	10.0	1.04 — 1.69	0.085
„ 18	5	*	**	0	1.33	1.24	6.8	1.13 — 1.40	0.080
„ 19	4	**	**	*	1.33	1.50	12.8	1.27 — 1.78	0.051
„ 20	5	*	**	0	—	1.39	—	1.03 — 1.88	0.121
„ 27	5	*	**	0	1.25	1.16	7.2	1.01 — 1.34	0.062
CATS									
Mar. 12	5	**	**	0	0.80	0.79	1.3	0.72 — 0.86	0.035
„ 31	4	**	**	0	0.87	0.80	8.0	0.70 — 0.92	0.049
May. 20	4	*	**	0	0.80	0.81	1.3	0.70 — 0.96	0.058
„ 21	4	0	**	0	—	1.00	—	0.83 — 1.15	0.064
„ 23	4	**	**	0	1.34	1.47	9.7	1.15 — 1.89	0.035

U = Strength of "unknown" solution.
S = Strength of ('standard" solution.
** = Highly significant (F found exceeded the tabulated value of F at the 1 % level of probability).
* = Significant (F was larger than F 5 %).
0 = Not significant (F found was smaller than F 5 %).

The sensitivity of both preparations was not yet specifically compared but from our results we may deduce that they are of the same *order of magnitude*, although the rat seems a little more sensitive. Howe-

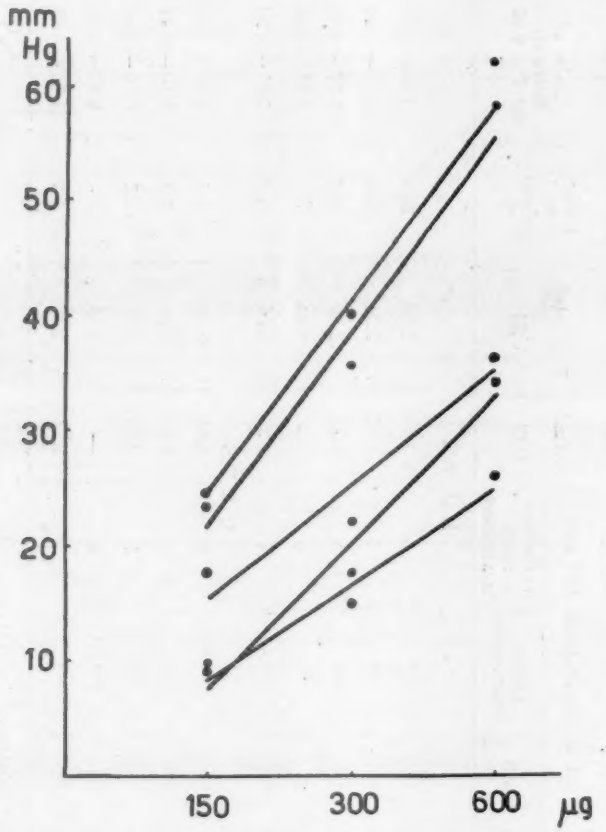

FIG. 3. — *Dose-response-relationship of EPI hypertensin in the pithed cat. Results obtained in 5 cats. Each point is the average of 4 injections of the same dose in the test animal and the lines were calculated by the method of the least squares. Ordinate: blood pressure rises in mm Hg; abscisa: EPI doses in micrograms.*

ver, as a consequence of the cat's larger precision (smaller λ), lesser percentage of casualties and because the time during which the preparation may be used is much longer, we are convinced that the cat is the preparation of choice. We have been able to use the rat preparation up to approxi-

mately 3 hours, while the cat has been used up to 6-8 hrs., and is then discarded, still in good conditions.

Discussion

We believe that the four point assay for hypertensin here introduced represents a definite improvement over the methods previously described.

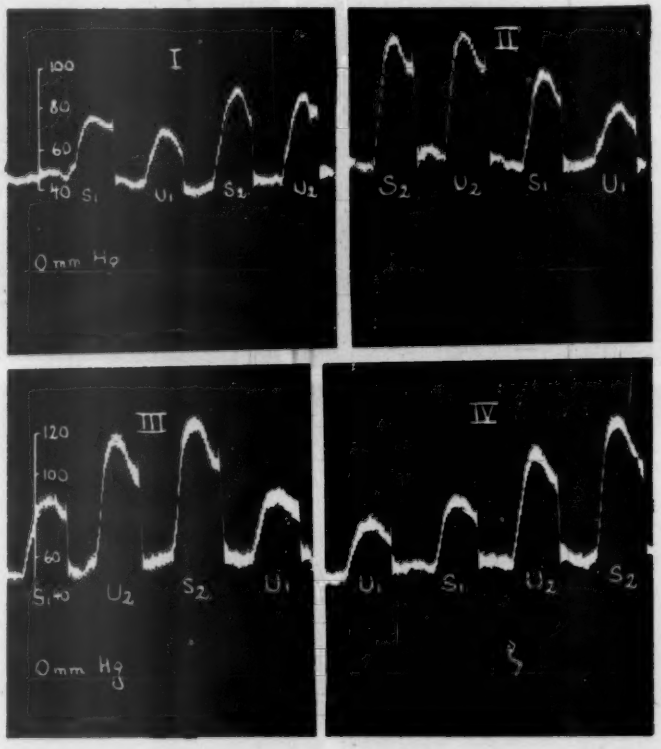

FIG. 4. — *Pithed cat's blood pressure. A typical 2 × 2 assay of Hypertensin. Effects of two doses of a "standard" hypertensin solution (S_1 and S_2) and two doses of a solution of "unknown" strength (U_1 and U_2). Time between injections 6 minutes, between groups 10 minutes.* $S_1 = 1.0$; $S_2 = 2.0$; $U_1 = 0.8$; $U_2 = 1.6$ *units of Helmer's Hypertensin.*

The difficulty which exists at present of assessing the actual potency of one hypertensin preparation in terms of another will probably be much ameliorated.

Of course the four-point assay described in this paper claims no specificity towards hypertensin and the identification of the substance to be assayed has to be previously secured from other pharmacological tests.

In this respect the use of the guinea-pig ileum and the rat's male genitals (Picarelli, Branco y Valle, 1951), are particularly valuable to exclude substances such as adrenalin, arterenol, serotonin, pitressin, simpatho-

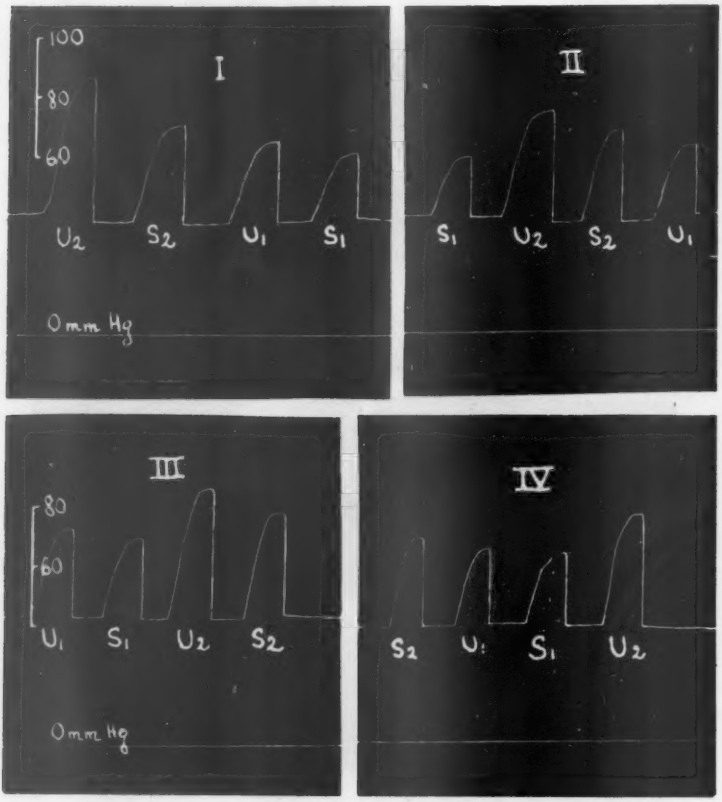

FIG. 5. — *Pithed rat's blood pressure. A 2 × 2 assay of Hypertensin. Effects of two doses of a "standard" hypertensin solution (S_1 and S_2) and two doses of a solution of "unknown" strength (U_1 and U_2). Time between injections, 6 minutes; between groups, 15 minutes.* $S_1 = 150$ μg; $S_2 = 300$ μg; $U_1 = 200$ μg; $U_2 = 400$ μg *of Edman's Hypertensin.*

mimetic amines, etc. The type, rapidity and duration of the response, however, may be used advantageously as a criterion to differenciate among various vasopressor substances.

TABLE 9

Exp. Nº	Ratio of potency (real)	Number of groups	Tests of significance			Ratio of potency (estimated)	Limits of estimation (99 %)	σ/b $(=\lambda)$
			Stand. x Unknown	Regress. line	Dev. from parallel.			
I	1.25	4	HS	HS	0	1.28	1.52 — 1.09	0.044
II	1.25	6	HS	HS	0	1.26	1.60 — 1.00	0.033
III	1.25	2	0	HS	0	1.22	1.71 — 0.86	0.074
IV	1.25	5	HS	HS	0	1.18	1.31 — 1.06	0.034
V	1.25	4 (L^2)	S	HS	0	1.25	1.66 — 0.94	0.079
VI	1.25	4 (L^2)	HS	HS	0	1.23	1.44 — 1.05	0.036

SUMMARY

Using pithed rats or pithed cats as test preparations, it was possible to demonstrate that within a sufficiently large zone there is a linear regression between the logarithm of the dose and the blood pressure increase produced by hypertensin.

Within this range of linearity it was possible to develop a four point assay for hypertensin both in pithed cats and pithed rats. Following the principles used by Schild (1942), two doses of a "standard" preparation (S_1 and S_2) having between them a certain relation (1:2, 1:1.5, etc.), and two doses of an "unknown" preparation (U_1 and U_2) keeping between them the same relation, are randomized and injected every 5-7 minutes. This *group* of doses is repeated four times, leaving 10-15 minutes of interval between groups. The analysis of variance of the results obtained permits the calculation of the relation U/S with a precision which is measured in each assay.

The sensitivity of both test preparations is of the same order of magnitude and the *precision* is better with the cat.

Acknowlegments. It is a pleasure to acknowledge the assistance from Dr. E. Chapadeiro in the performance of some assays and from Dr. A. M. Penha in the statistical analysis of the material.

REFERENCES

BRAUN-MENÉNDEZ, E., FASCIOLO, J. C., LELOIR, L. F. MUÑOZ, J. M., TAQUINI, A. C.: *Renal hypertension*, Springfield, Ill, Charles C. Thomas, 1946.

BURN, J. H.: *Biological standardization*, 2nd. ed., Oxford University Press, London, 1950.

EMMENS, C. W., *Principles of biological assay.* Chapman and Hall Ltd., London, 1948.
FASCIOLO, J. C., TAQUINI, A. C.: *Rev. Soc. Argent. Biol.*, 1947, *23*, 138.
HOLTON, P.: *Brit. J. Pharmacol.*, 1948, *3*, 328.
PAGE, I. H.: *J. Lab. Clin. Med.*, 1942, *23*, 336.
PICARELLI, Z. P., BRANCO, T. S., VALLE, J. R.: *Ciencia e Cultura*, 1951, *3*, 288.
ROCHA E SILVA, M.: *Seminários de Estatistica Aplicada*, 3ª série, 59-70. Published by Divisão de Experimentaçao e Pesquisas, Instituto Agronômico, Campinas, São Paulo.
ROCHA E SILVA, M.: *Bradicinina, Preparo, ensaio biológico, identificação.* Tese apresentada à Faculdade Nacional de Medicina da Universidade do Brasil, 1951.
SCHILD, H. O.: *J. Physiol.*, 1942, *101*, 115.
SHIPLEY, R. E., HELMER, O. M., KOHLSTAEDT, K. G.: *Amer. J. Physiol.*, 1947, *149*, 708. 708.
SHIPLEY, R. E., TILDEN, J. H.: *Proc. Soc. Exper. Biol.*, N. Y., 1947, *64*, 453.
VALLE, J. R., HENRIQUES, S. B., HENRIQUES, O. B.: *Endocrinology*, 1947, *41*, 335.